EXHIBIT A

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Is vaccination against transmissible spongiform encephalopathy feasible?

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Summary

Prion diseases are a unique category of illness, affecting both animals and humans, where the underlying pathogenesis is related to a conformation change of the cellular form of a normal, self-protein called a prion protein (PrP^{C} [C for cellular]) to a pathological and infectious conformation known as scrapie form (PrP^{Sc} [Sc for scrapie]). Currently, all prion diseases are without effective treatment and are universally fatal. The emergence of bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease has highlighted the need to develop possible therapies. In Alzheimer's disease (AD), which has similarities to prion diseases, both passive and active immunisation have been shown to be highly effective at preventing disease and cognitive deficits in model animals. In a human trial of active vaccination in AD, despite indications of cognitive benefits in patients with an adequate humoral response, 6% of patients developed significant complications related to excessive cell-mediated immunity. This experience highlights that immunotherapies designed to be directed against a self-antigen have to finely balance an effective humoral immune response with potential autoimmune toxicity. Many prion diseases have the gut as a portal of infectious agent entry. This makes mucosal immunisation a potentially very attractive method to partially or completely prevent prion entry across the gut barrier and to also produce a modulated immune response that is unlikely to be associated with any toxicity. The authors' recent results using an attenuated *Salmonella* vaccine strain expressing the prion protein show that mucosal vaccination can partially protect against prion infection from a peripheral source, suggesting the feasibility of this approach.

Keywords

Bovine spongiform encephalopathy – Chronic wasting disease – Conformational disorder – Mucosal vaccine – Prion – Salmonella – Transmissible spongiform encephalopathy – Variant Creutzfeldt-Jakob disease.

Introduction

Prion disease occurs both in humans and in various animals such as cows, sheep, goats, mink, deer and elk. These diseases are also known as transmissible spongiform encephalopathies or prionoses. They are a unique category of illness in that they can be infectious or transmitted genetically and are sporadic in occurrence. Abundant evidence has made it clear that these slow infections are neither caused by a virus nor any nucleic acid containing particle. A comprehensive body of evidence has presented compelling data that the transmissible pathogen for these diseases is a proteinaceous infectious particle (hence the term 'prion') (37, 38). All prion diseases result from a conformational alteration of the same host-derived prion protein (PrP^{C} [C for cellular]) to a disease-associated conformer called PrP^{Sc} (Sc for scrapie). This conversion can be precipitated by an exogenous, infectious source of PrP^{Sc}, a mutation in the prion protein that predisposes to such a conformational change, or a spontaneous conformational change, as occurs in sporadic prion disease.

The human forms are kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia. In animals these diseases include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk and transmissible mink encephalopathy (42). Neuropathologically, these different forms of the disease are all characterised by spongiform change, neuronal loss and astrocytosis; in addition amyloid deposition may occur. However, the regional pattern of brain lesions and the extent of prion amyloid deposition vary within and between species. Within species, these differences depend on the strain of prion causing the infection. A barrier exists limiting transmission of prions across species, but once this barrier is overcome a new, stable and distinct pattern of infection can develop in the new host species.

Bovine spongiform encephalopathy, variant Creutzfeldt-Jakob disease and chronic wasting disease

Interest in prion disease has greatly increased since the emergence of BSE in the United Kingdom (UK) and the resulting appearance of variant CJD (vCJD) in human populations. Bovine spongiform encephalopathy arose from the feeding of cattle with prion-contaminated meat and bone meal products, while vCJD developed following entry of BSE into the human food chain (8). Since the original report in 1996 (60) a total of 182 confirmed cases of vCJD have been diagnosed, 156 in the UK, 17 in France, 3 cases in Ireland and one each in Italy, Canada, Japan, the Netherlands, Saudi Arabia and the United States of America (USA). The patients from these countries resided in the UK during a key exposure period of the population to the BSE agent. It has been difficult to predict the expected future numbers of vCJD. Mathematical analysis has predicted that between 1,000 and 136,000 individuals will eventually develop the disease. This broad range reflects a lack of knowledge regarding the time of incubation and the number of patients who could be infected from a given dosage of BSE agent. Because the vCJD agent is present at high levels in the lymphatic tissue, screening for PrP^{Sc} was performed on sections from lymph nodes, tonsils, and appendices taken from archives in the UK. Three out of 12,674 randomly selected samples showed evidence of subclinical infection, leading to a prediction that about 4,000 further cases of vCJD may occur in the UK. However, there is much uncertainty about such a predication, as it is not known if all subclinical infections will progress or whether such screening of lymphoid tissue would capture all subclinical cases. The initially predicted epidemic of vCJD does not seem to be materializing, as the number of cases in the UK has declined from a peak of 28 in 2000 to 17 in 2002, with only 5 cases in 2005 (8). A complicating factor for estimating future numbers of vCJD is the occurrence of several transfusion-associated cases. These occurred after incubation periods of 6 to 8 years. One of these disease-associated donations was made more than 3 years before the donor became symptomatic, suggesting that vCJD can be transmitted from silently infected individuals (11). The estimated risk for new cases of vCJD in other European countries is much lower. In the UK, 200,000 cases of BSE were reported (it is estimated that four times this number entered the food chain), compared to a combined total of approximately 500 BSE cases in other European countries. This suggests a significantly lower exposure of these populations to BSE prions. A few cases of BSE have also been reported in other parts of the world, such as Japan, the USA and Canada.

Of greater concern in North America is CWD. This disease is now endemic in Colorado, Wyoming and Nebraska and continues to spread to other parts of the USA. Cases have been reported in the Midwest and it has now been detected as far east as New York State (61). Most vulnerable to CWD infection are white-tailed deer, and the disease is now found in areas with large populations of these animals, which indicates that its prevalence can be expected to increase substantially in the future. Occurrence of CJD among three young deer hunters raised speculation that CWD could be transmitted to humans (7), but autopsy of these three subjects did not show the extensive amyloidosis characteristic of vCJD and CWD (25). However, like BSE, CWD is transmissible to non-human

primates and transgenic mice expressing human PrP^C (41, 54, 58). Therefore, the possibility of such transmission needs to be closely monitored. Chronic wasting disease is similar to BSE in that the peripheral titres of the prion agent are high. PrP^{Sc} has been detected in both the muscle and saliva of CWD-infected deer (1, 30).

Biology of the prion protein

PrP^C is expressed in many types of cells; however, the highest level of expression is found in central nervous system (CNS) neurons (21, 24). A knowledge of the molecular anatomy of PrP^C is crucial for understanding its malfunction in prion diseases. The whole protein is located on the outer surface of the cell anchored to the cell membrane by phosphotidylinositol glycolipid (GPI) attached to its C-terminus. The central portion of the peptide contains one short α-helical segment (α-helix A) flanked by two short β-strands. The N-terminus is unstructured and extends into the intracellular space. The N-terminus harbours five octapeptide repeats. Histidines located within the octapeptides bind copper ions (9). It has been postulated recently that the possible function of PrP^C is to capture, store, and present copper to the neuron (9, 39, 40). The copper binding state of PrP^C influences its conformation and copper chelation has been shown to inhibit PrP^{Sc} infection (48). The exact function of PrP^C remains to be elucidated. The protein is not essential since Prnp knock-out mice (12) did not show a significant disease phenotype. Minor abnormalities in synaptic physiology (14) and in circadian rhythm (55) have been described in these knock-out mice.

Prion diseases and other conformational disorders

The prion diseases belong to a broader category of conformational diseases (43). The etiology of each of the conformational diseases is related to a specific protein that can exist in at least two distinct forms associated with either health or disease. The most common conformational disorder is Alzheimer's disease (AD), in which the disease state is associated with the accumulation of an endogenously expressed peptide, the amyloid-β peptide, in a β-sheet structure within neuritic plaques. Other conformational disorders include Parkinson's and Huntington's diseases. The pathological conformer of PrP^C is PrP^{Sc}, which due to its increased β-sheet content demonstrates increased resistance to proteolysis and the ability to aggregate and polymerize. Although the insolubility of PrP^{Sc} has prevented crystallographic conformational studies, less exact structural methods such as circular dichroism and Fourier transform infrared spectroscopy indicate a β-sheet content as high as 45% (compared with 3% in PrP^C) and a α-helix content of 30% (40% in PrP^C) (3).

Understanding the mechanism that converts PrP^C into PrP^{Sc} is another intriguing aspect of prion diseases. One of the most crucial features of PrP^{Sc} is its ability to bind to PrP^C; this initiates a self-perpetuating vicious cycle and enables prion diseases to be transmitted (38). It has been demonstrated in cellular models that the PrP is transported to the membrane in the PrP^C form and that the conversion of PrP^C to PrP^{Sc} occurs at the cell surface. Neurons produce native PrP^C (24) and transport it to the cellular surface where it can encounter PrP^{Sc}, leading to its conformational change into a high β-sheet content state. During progression of the disease, the amount of PrP^C produced remains stable, whereas the amount of PrP^{Sc} increases. The homozygosity of PrP^C facilitates prion replication. This has been observed in humans with respect to the codon 129 polymorphism, as well as in sheep with respect to the VRQ/VRQ polymorphisms. Evidence from transgenic animals expressing various segments of PrP^C indicates that residues 90-150 are required for the interaction with PrP^{Sc} leading to conversion of PrP^C into PrP^{Sc}. The spontaneous conversion of PrP^C into PrP^{Sc} has been demonstrated in sheep and probably is the major cause of scrapie and sporadic CJD.

The immune system and prion infection

The prion protein is a self-antigen; hence, prion infection is not known to elicit a classical immune response. In fact, the immune system is involved in the peripheral replication of the prion agent and its ultimate access to the CNS (4, 50). Paradoxically, immune suppression with, for example, splenectomy or immunosuppressive drugs, increases the incubation period. This incubation period, during which time the prion agent replicates peripherally without producing any symptoms, is quite long, lasting many months in experimental animals and up to 56 years in documented human cases associated with cannibalistic exposure to the prion agent (15). Lymphatic organs such as the spleen, tonsil, lymph nodes or gut-associated lymphoid tissue (GALT) contain high concentrations of PrP^{Sc} long before PrP^{Sc} replication starts in the brain (10, 26). Cells found to be particularly important for peripheral PrP^{Sc} replication are the follicular dendritic cells and the migratory bone-marrow derived dendritic cells (5, 26). Dendritic cells from infected animals are capable of spreading the disease (5). An emerging therapeutic approach for prion infection is immunomodulation (44, 50).

Vaccination for prion infection

Currently there is no treatment that would arrest and/or reverse progression of prion disease in non-experimental settings, although many approaches have been tried (56). In AD model mice it has been definitively shown that immunotherapy can prevent the onset of cognitive deficits and the development of amyloid lesions (31, 63). Significantly, this method of treatment is associated with consistent cognitive benefits in the mice (2, 20, 32, 49). An antibody-mediated response is probably critical for a therapeutic response, since similar results have been obtained with passive immunisation (6). Active immunisation for AD has recently been tried in humans by Elan Pharmaceuticals, with significant toxicity resulting from the vaccine (18, 62, 63). In the human phase 2A clinical trial of the vaccine (called AN-1792) 18 out of 372 patients worldwide developed symptoms of meningitis or meningoencephalitis, with symptoms apparently responding to immunosuppression in most patients (12 patients out of the 18 responded fully) (18). Recent evidence suggests that patients who developed anti-Aβ titres benefited cognitively from vaccination, including patients among the 12 that initially had complications (18, 19) and that vaccination resulted in amyloid clearance as judged by three autopsies performed in vaccinated patients (two autopsies from patients with encephalitis and one without complications) (17, 28, 33). Hence, it appears that if safety issues can be addressed, a vaccine approach will prove to have important therapeutic value in patients (58, 63) and it is the subject of new ongoing trials.

In part because of this success in AD models, similar experiments with anti-PrP antibodies were initiated in prion infectivity culture models and active and passive immunisation studies were carried out in rodent models. Earlier *in vivo* studies had shown that infection with a slow strain of PrP^{Sc} blocked expression of a more virulent fast strain of PrP, mimicking vaccination with a live attenuated organism (27). In tissue culture studies anti-PrP antibodies and antigen binding fragments directed against PrP have been shown to inhibit prion replication (16, 34, 35). One study demonstrated that active immunisation with recombinant PrP delayed the onset of prion disease in mice, but the therapeutic effect was relatively modest and eventually all the mice succumbed to the disease (46). This limited therapeutic effect may be explained by the observation that antibodies generated against prokaryotic PrP often do not have a high affinity towards PrP^{C} (36), although in studies carried out by the authors the increase in the incubation period correlated well with the antibody titres against PrP^{C}. The follow-up passive anti-PrP immunisation study confirmed the importance of the humoral response, showing that anti-PrP antibodies are able to prolong the incubation period (47). Subsequently, other investigators, using a much higher antibody dosage, were able to completely prevent disease onset in mice exposed to PrP^{Sc}, provided passive immunisation was initiated within a month of exposure (59). This type of approach could be used immediately following accidental exposure in humans to prevent future infection. However, passive immunisation has not been found to be effective closer to the clinically symptomatic stages of prion infection. Moreover, passive immunisation would be too costly an approach for animal prion diseases.

In the development of immunotherapeutic approaches targeting a self-antigen, designing a vaccine avoiding autoimmune related toxicity is a major concern. The emerging data from AD-targeting immunisation is that toxicity is due to excessive cell-mediated immunity within the CNS, while the therapeutic response is linked to humoral immunity. In addition, toxicity could be partially related to the immunogen and/or to the adjuvant used; in the human AD vaccination trial fibrillar Aβ1-42 was used as an immunogen. This peptide is well known to be toxic. Hence, the authors have been promoting the use of nonamyloidogenic derivatives as immunogens for protein conformational disorders, including AD and prion diseases (45, 49, 63) and interestingly a recent study indicated that α-helical PrP elicited an antibody response whereas an amyloidgenic β-sheet form of PrP favored a cytotoxic T-cell response (51). How significant an issue direct toxicity of the immunogen may be for prion vaccination remains unclear. Unlike the amyloid β peptide used for vaccination in AD models, direct application of recombinant PrP has not been shown to be toxic. However, this issue has not been investigated as thoroughly as in the Alzheimer's field. One study has shown that cytosolic accumulation of PrP was toxic (52), whereas other investigators observed that PrP was neuroprotective in another cell culture model (22).

A potential ideal means of using immunomodulation to prevent prion infection is mucosal immunisation. One important reason for this is that the gut is the major route of entry for many prion diseases such as CWD, BSE and vCJD. Furthermore, mucosal immunisation can be designed to induce primarily a humoral immune response, avoiding the cell-mediated toxicity that was seen in the human AD vaccine trail. Recently, the authors have been developing prion vaccines that target gut-associated tissue, the main site of entry of the prion agent. One of their approaches is to express PrP in attenuated *Salmonella* strains as a live vector for oral vaccination. Live attenuated strains of *Salmonella enterica* have been used for many years as vaccines against salmonellosis and as a delivery system for the construction of multivalent vaccines, with broad applications in human and veterinary medicine (29). One of the main advantages of this system is that the safety of administering live attenuated *Salmonella* has been extensively confirmed in humans and animals (23, 53).

Ruminants and other veterinary species can be effectively immunised by the oral route using live *Salmonella*, to induce humoral mucosal responses (13, 57). The authors are currently exploring ways to increase the efficacy even further. In these studies, the mucosal IgA anti-PrP titre correlates well with the delay or prevention of prion infection, further supporting the importance of the humoral response for the therapeutic effect. *Salmonella* target M-cells, antigen sampling cells in the intestines, which may also be important for uptake of PrP^{Sc} (26, 50). Hence, this approach is more targeted than prior vaccination studies, which probably explains the improved efficacy. By exploring other strains of attenuated *Salmonella*, using different bacteria or oral adjuvants, and/or by altering the expression levels or sequence of the PrP antigen, it is likely that the percentage of uninfected animals can be improved. The authors' recent work utilising this approach indicates that complete protection to clinical prion infection via an oral route is possible. Overall, this approach holds great promise as an inexpensive prophylactic immunotherapy to prevent the spread of prion disease, particularly in animals at risk and perhaps eventually in certain high-risk human populations.

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La vaccination contre l'encéphalopathie spongiforme transmissible est-elle une option réaliste ?

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Résumé

Les maladies à prion constituent une catégorie unique de pathologies affectant aussi bien les animaux que l'homme et dont la pathogénèse est associée à une conversion de la protéine de l'hôte, appelée protéine prion, de sa forme cellulaire normale PrP^{C} (C pour cellulaire) en une conformation pathogène et infectieuse appelée PrP^{Sc} (Sc pour *scrapie*, tremblante en anglais). À l'heure actuelle, il n'existe aucun traitement efficace contre les maladies à prion, dont l'issue est toujours fatale. L'émergence de l'encéphalopathie spongiforme bovine et de la variante de la maladie de Creutzfeldt-Jakob exige la mise au point de nouveaux traitements. Dans des expérimentations portant sur la maladie d'Alzheimer (qui présente des similitudes avec les maladies à prion), l'immunisation passive et active s'est révélée efficace pour prévenir la maladie chez les animaux de laboratoire et pour limiter les troubles cognitifs qui en résultent. Lors d'une série d'essais de vaccination active contre la maladie d'Alzheimer chez l'homme, une amélioration des fonctions cognitives a été obtenue chez des patients présentant une bonne réponse humorale, mais 6 % des patients ont souffert de complications graves, liées à une réponse à médiation cellulaire trop importante. Cette expérience met en exergue la nécessité, dans le domaine des immunothérapies dirigées contre un antigène autologue, de parvenir à un difficile équilibre entre la recherche d'une immunité humorale et le souci d'éviter toute toxicité auto-immune. Pour de nombreuses maladies à prion, l'intestin est l'organe par où l'agent pathogène pénètre dans l'organisme. De ce fait, l'immunisation muqueuse est une méthode particulièrement prometteuse qui vise à empêcher totalement ou partiellement le prion de franchir la paroi intestinale tout en produisant une réponse immunitaire ciblée et exempte de toxicité. Les résultats obtenus par les auteurs

en utilisant une souche vaccinale atténuée de *Salmonella* exprimant la protéine prion montrent que la vaccination muqueuse confère une protection partielle contre l'infection à prion à partir d'une source périphérique, ce qui paraît confirmer la faisabilité de cette démarche.

Mots-clés
Cachexie chronique – Encéphalopathie spongiforme bovine – Encéphalopathie spongiforme transmissible – Immunisation mucosale – Prion – Salmonella – Trouble de la conformation – Variante de la maladie de Creutzfeldt-Jakob.
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¿Es factible la vacunación contra la encefalopatía espongiforme transmisible?

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Resumen
Las enfermedades priónicas constituyen una singular categoría de dolencias que afectan tanto a los animales como al hombre y cuya patogénesis guarda relación con el cambio de conformación de una proteína del propio organismo, que pasa de la llamada forma celular (PrP^{C} [proteína priónica celular]) a una conformación patológica e infecciosa denominada forma priónica (PrP^{Sc} [en inglés, "*scrapie form*"]). En la actualidad no hay tratamiento eficaz para ninguna de esas enfermedades, que resultan invariablemente fatales. La aparición de la encefalopatía espongiforme bovina y de la variante de la enfermedad de Creutzfeldt-Jakob ha hecho más necesario que nunca encontrar posibles terapias. En el caso de la enfermedad de Alzheimer, que presenta similitudes con las afecciones priónicas, se ha demostrado que en modelos animales la inmunización tanto pasiva como activa resulta muy eficaz para prevenir la enfermedad y las consecuentes deficiencias cognitivas. En el curso de un ensayo de vacunación activa contra la enfermedad realizado en seres humanos, y pese a ciertos signos que indicaban beneficios cognitivos en pacientes con una buena respuesta humoral, se observaron importantes complicaciones ligadas a una respuesta excesiva de inmunidad celular en un 6% de los pacientes. Esa experiencia pone de manifiesto que las terapias inmunológicas dirigidas contra un autoantígeno deben hallar un delicado equilibrio entre la búsqueda de eficacia de la respuesta inmunitaria humoral y el riesgo de toxicidad autoinmune. En muchas enfermedades priónicas el intestino es la vía de entrada del agente infeccioso, lo que hace de la inmunización de las mucosas un método en potencia muy atractivo para prevenir, parcial o totalmente, la penetración de un prión a través de la barrera intestinal y también para inducir una respuesta inmunitaria modulada poco susceptible de generar toxicidad. Los resultados obtenidos recientemente por los autores (con una cepa vacunal de salmonelas atenuadas que expresan la proteína priónica) demuestran que la inmunización de las mucosas puede conferir protección parcial contra las infecciones priónicas procedentes de una fuente periférica, lo que lleva a suponer que se trata de un método viable.

Palabras clave
Anomalía de conformación – Caquexia crónica – Encefalopatía espongiforme bovina – Encefalopatía espongiforme transmisible – Inmunización de mucosas – Prión – Salmonella – Variante de la enfermedad de Creutzfeldt-Jakob.
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EXHIBIT B

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SHORT ANALYTICAL REVIEW

Prion Diseases and the Immune System

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Transmissible spongiform encephalopathies are caused by unusual infectious agents that are purported to contain a single type of macromolecule, a modified host glycoprotein. The term prion has been applied to this group of agents. Surprisingly, the immune system appears to behave as a Trojan's horse rather than a protective fortification during prion infections. Because prions seem to be essentially composed of a protein, PrP^{Sc}, identical in sequence to a host encoded protein, PrP^{C}, the specific immune system displays a natural tolerance. However, lymphoid organs are strongly implicated in the preclinical stages of the disease. Certain immunodeficient animals are resistant to prions after peripheral inoculation. In normal subjects, cells of the immune system support the replication of prions and/or allow neuroinvasion. A better understanding of these aspects of prion diseases could lead to immunomanipulation strategies aimed at preventing the spread of infectious agents to the central nervous system. © 2000 Academic Press

Key Words: **PrP; lymphoid organs; immune tolerance.**

INTRODUCTION

Concern regarding the transmissible spongiform encephalopathies (TSE) has been raised by recent evidence that a new form of Creutzfeldt–Jakob disease (variant Creutzfeldt Jakob disease, vCJD) is related to bovine spongiform encephalopathy (BSE, or "mad cow" disease). Regardless of the future incidence of vCJD, the discovery of an unusual type of infectious agent whose replication mechanisms may involve new biological concepts provides a considerable challenge for medical researchers. Furthermore, since human intervention (feeding cows with animal-derived products) has lead to the emergence of BSE and vCJD, it is possible that other TSEs will emerge as a function of human activity.

TSEs include scrapie in sheep, goats, and experimental animals, BSE, and Creutzfeldt–Jakob disease and kuru in humans (1, 2). The causative agent, the prion, seems to be mainly composed of a protein, PrP^{Sc}. Prions are said to replicate in the central nervous system and some other organs of the host by inducing a posttranslational modification of the endogenous physiological form of the prion protein, PrP^{C}, to the pathological form PrP^{Sc} (3–8). Experimental models have been developed in laboratory animals, including Syrian hamsters and mice, following the passage of agents found in naturally occurring diseases in humans, mink, cattle, and sheep (9–11).

Many aspects of prions make them unusual infectious agents. The most interesting, although still controversial, new concept is that these infectious particles seem to lack any nucleic acid, and their pathogenic potential is enciphered only in the conformation of PrP^{Sc}, which would be able to induce the transconformation of its host physiological isomer, PrP^{C}, into PrP^{Sc} (8). This "protein-only" hypothesis has been questioned because of the existence of numerous strains of agents with distinct properties, suggesting that additional information is borne by another component of the infectious particles (12) and by the demonstration that BSE can be experimentally transmitted to mice without formation of PrP^{Sc} (13).

A second peculiarity, which although remarkable has drawn less attention until recently, is the unusual role of the immune system. It has long been observed that there is no specific humoral and cellular immune response against prions (14, 15). Moreover, the immune system appears to help rather than impair the propagation of prions: Although PrP^{C} is expressed at various levels both in and outside the brain (16), it is worth noting that there is no evidence that infectivity multiplies in tissues other than the nervous system and the peripheral lymphoid organs.

THERE IS NO SIGNIFICANT SPECIFIC IMMUNE RESPONSE AGAINST PRIONS

No antibody against prions can be detected in experimentally infected animals or TSE-affected patients (14, 17). Mixed lymphocyte cultures of splenocytes

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from normal and scrapie mice also failed to reveal any detectable specific cellular response to prions, although the T-cell functions were globally unaffected (15). On the other hand, macrophages were shown to capture the scrapie agent *in vitro* (18) and seem to be able to partially inactivate it (19). A possibility could be that the unusual protease resistance of PrP^{Sc} prevents it from being processed into peptides by antigen-presenting cells. However, it seems that prions have normal immunogenic properties: mice in which the gene encoding PrP has been knocked out ($Prnp^{0/0}$) are able to mount a normal humoral immune response when PrP is injected with Freund's adjuvant (20, 21); an immune response is not observed when $Prnp^{0/0}$ mice are inoculated with scrapie prions alone as part of an infection protocol, probably because of their inability to propagate the prions (22). Successful immunizations of $Prnp^{0/0}$ mice were also achieved by intramuscular injections of DNA plasmids encoding human PrP (23). Immunization of mice with prions from scrapie-infected hamster brains allowed antibodies to be obtained which bind hamster but not mouse PrP (24). A discrete impairment of B-cell response to lipopolysaccharide has been found in scrapie-infected mice (25). Increased serum IgG levels in scrapie sheep and mice might reflect anomalies related to the involvement of lymphoid organs (26, 27); however, most studies show that normal immune functions are not affected by prion infection (14, 28), suggesting that the unresponsiveness to prions is not related to an induced immunodeficiency. The absence of a specific immune response may be explained by the absence of specific T-cell help, due to immunological tolerance toward all PrP-related peptides that can be processed by antigen-presenting cells. Whether tolerance is complete or partial, i.e., directed to only a few immunodominant epitopes, remains to be elucidated. The identification of nontolerized cryptic epitopes could be of great importance in the perspective of generating immune responses against infected cells.

PRIONS ACCUMULATE IN PERIPHERAL LYMPHOID ORGANS, WITHOUT PATHOLOGY

Although the prion diseases are neurological conditions, critical events in their pathogenesis take place in restricted sites out of the nervous system, especially in peripheral lymphoid organs (5). Lymphoid organs have long been known to be involved in early steps of prion diseases (29–32). In particular, the spleen and lymph nodes have been demonstrated to be the first sites of PrP^{Sc} replication after infection by peripheral routes and to also be significantly affected following intracerebral challenge. In humans, abundant PrP^{Sc} was demonstrated in the germinal centers of tonsils from a patient affected with vCJD (33) and in the appendix before clinical onset in another case (34).

The natural history of the disease has been described primarily in experimental animal models (29–32, 35–37). These early studies of sequential organ involvement led to hypotheses concerning the probable critical preclinical steps and the issue of how the infectious agent might reach the nervous system. Whether these studies are relevant to the pathophysiology of human TSE is difficult to affirm; also, different prion strains might behave differently.

Since prion diseases are naturally transmitted by peripheral routes, either orally or transcutaneously, the intracerebral inoculation commonly used for studies on brain pathology has little relevance regarding the early stages of the natural disease. It is worth noting, however, that the relative efficiencies of intracerebral and peripheral routes are strikingly different: up to 10,000 more inoculum may be required by subcutaneous compared with intracerebral injection, and the intraveinous route is more efficient than intraperitoneal and subcutaneous inoculations (32); furthermore, the infectivity of certain prions may be significantly different according to the sites of subcutaneous injection (32). Inoculation of a prion strain adapted from sheep to mice (known as the Chandler strain) by subcutaneous injection showed that the agent first accumulates in the spleen and lymph nodes (29) (Fig. 1). Four weeks after injection, these organs were shown to contain titers of infectivity greater than the inoculum, corresponding to newly formed infectivity, with the maximum being reached at 8 weeks. Similar levels were detected in the brain 16 weeks following inoculation. Early involvement of lymphoid organs, especially the spleen, was confirmed after intraperitoneal and intraveinous infections (32).

Replication of prions in lymphoid organs was also demonstrated after intracerebral inoculation, raising the question of a role in advanced stages of the disease (32). Severe combined immunodeficient (SCID) mice are resistant to infection by the BSE agent, even after intracerebral challenge (38), which suggests that lymphoid cells could favor the trespassing of species barriers. Other interesting experiments revealed that, although mice that lack B cells do not develop neurological signs of scrapie infection after peripheral challenge (39), they may nevertheless accumulate PrP^{Sc} and infectivity in the brain (40); thus, the clinical expression of the disease might partly rely upon certain aspects of the immunological status.

INVOLVEMENT OF LYMPHOID ORGANS IS CRITICAL FOR PRION INFECTION BY THE PERIPHERAL ROUTE

Several indirect pieces of evidence suggest that the lymphoid system influences the course of TSE (Table

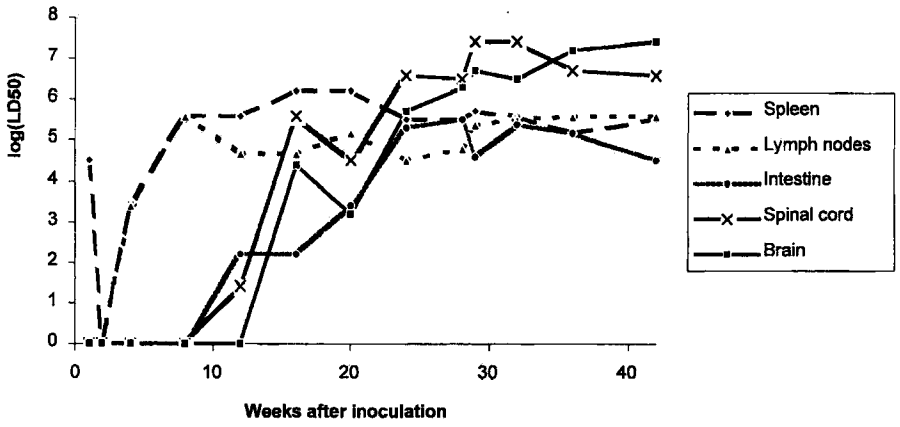

FIG. 1. Evolution of infectivity titers in several organs from mice inoculated with prions (after Eklund *et al.* (29)).

1). Susceptibility to prion infection correlates with the maturation of the immune system in ontogeny (41). Corticosteroids reduce the susceptibility to scrapie (42). Mitogenic stimulation of lymphoid cells enhances the susceptibility to scrapie (43). Splenectomy significantly delays the onset of clinical symptoms in mice infected by the peripheral route, while it does not affect the incubation period after intracerebral infection (30, 31). Finally, studies on mice with defined immunodeficiencies demonstrate with more precision the role of the immune system in the pathophysiology of TSE. Mice with severe combined immunodeficiency are partially resistant to scrapie after intraperitoneal or subcutaneous inoculation, in contrast to immunocompetent mice of the same strain and immunologically reconstituted SCID mice (44, 45).

The nature and actions of the immune cell type(s) which supports this initial step of infection still remain unclear. The essential controversy concerns the respective roles of lymphocytes and follicular dendritic cells (FDC) in the replication of prions and their transport to the nervous system. After fractionation of spleen cells by density gradient centrifugation, the highest infectivity between the second and ninth weeks following mice inoculation with scrapie agent was found in lymphocytes (46). However, it may be that this cell fraction also contained FDC because of their tight interactions with B lymphocytes. Ionizing radiations did not influence mouse susceptibility to scrapie, suggesting that quiescent cells such as FDC might principally support prion replication (47). Because the scrapie susceptibility of SCID mice after reconstitution with hematopoietic precursors appears to be dependent on the restoration of a normal lymphoid architecture (44), and PrP^{Sc} is detected in FDC of CJD-infected mice (48), FDC are suggested to be involved in the replication and accumulation of the scrapie agent. However, the presence of the scrapie agent in FDC could reflect their potent antigen-capturing function (49). TNF-receptor-1 knockout mice, which lack FDC and germinal center reaction, have a normal susceptibility to scrapie after peripheral inoculation, while mature B-lymphocytes, rather than other hematopoietic cell lineages, are required for the neuroinvasion (39); however, such results were not confirmed with another model of FDC deficiency, the TNF-α knockout mouse (50). A key role of FDC in PrP^{Sc} replication is strongly supported by recent results showing that the expression of PrP^{C} by FDC, but not by hematopoietic lineages, is required for scrapie susceptibility after peripheral inoculation (50).

RAG-1 or RAG-2 knockout mice, which are deficient in T- and B-cells, do not develop disease after peripheral inoculation (39). A crucial role of the B-cell was suggested by a similar resistance of μMT mice, which bear a selective B-cell differentiation defect due to a targeted disruption of the immunoglobulin μ-chain transmembrane exon. Infectivity in the spleens could not be detected in these animals more than 500 days after peritoneal inoculation (39). The requirement of B-cells for neuroinvasion seems independent of the expression of the antibody repertoire, since transgenic t11μMT mice, in which B-cell differentiation is restored by the expression of a single rearranged heavy chain, are normally susceptible to prion infection. On the other hand, mouse strains partially or totally deficient in T-cells ($CD4^{0/0}$, $CD8^{0/0}$, β2-microglobulin$^{0/0}$, per-

TABLE 1

Effect of Immune System Functional Status on Mouse Susceptibility to Scrapie

	Incubation time	References
Neonatal immaturity	Increased	(41)
Corticosteroid treatment	Increased	(42)
Splenectomy	Increased	(30, 31)
Ionizing radiation	Unchanged	(47)
Mitogenic stimulation (PHA, LPS)	Decreased	(43)

forin$^{0/0}$ (39), and TCR$\alpha^{0/0}$ (40)) showed normal susceptibility to prion infection (Table 2). Such findings are in accordance with the results of a previous study, in which a fraction corresponding to splenic B-cells, especially low-density lymphocytes, was found to have a higher infectivity than a T-cell-enriched fraction (46). Although these models provide strong evidence for a critical and specific role of B-lymphocytes in PrP^{Sc} invasion from the periphery, the mechanisms have not yet been identified at the cellular and molecular levels. More recent results demonstrated that, while B-cells are required for susceptibility to scrapie after peripheral inoculation, they do not need to express the PrP gene, which strongly questions the hypothesis that B-cells would be a necessary site of prion replication (51) (Table 3). In view of the results obtained in chimeric mouse models with Prnp$^{0/0}$ hematopoietic cells or FDC (50), the requirement of B-cells for scrapie susceptibility could merely reflect their role in FDC maturation (52).

The competence of cells of lymphoid organs to replicate prions has been addressed with transgenic mice expressing PrP under the control of different tissue-specific promotor/enhancer sequences (53). When PrP^{C} is expressed only in lymphoid organs, these mice prove able to replicate PrP^{Sc} while the brain remains uninfected 6 months after inoculation. Specific expression of PrP^{C} by T-cells or by hepatocytes does not allow prion replication, suggesting that additional tissue-specific factors are required. Recent results from the same group reveal that, in spleens from scrapie-infected mice, infectivity is borne by T-cells, B-cells, and stromal cells, but not by monocytes/macrophages and granulocytes (54); transfer experiments showed that this infectivity cannot be aquired by B- or T-cells that do not express PrP^{C}. A likely hypothesis is that prions are provided by FDCs to lymphocytes through a specific interaction which is dependent on the presence of PrP^{C} (54). Of note, these recent results are difficult to conciliate with the ability of lymphoid precursor that do not express PrP^{C} to restore scrapie sensitivity in mice (51).

WHAT DO WE KNOW ABOUT THE INTERACTIONS OF PRIONS WITH THE LYMPHOID SYSTEM?

Outside of the nervous system, the apparent special targeting of lymphoid organs suggests that a specific factor present in lymphoid cells might support prion replication. Indeed, although the differentiation of bone marrow precursors to lymphoid cells, but not to granulocytes, is featured by increased expression of PrP^{C} (55), the presence of PrP^{C} may not be sufficient. A number of proteins that interact with PrP^{Sc} have already been reported, including chaperone proteins such as GroEL and HSP104, a member of the amyloid precursor protein family, the laminin receptor precursor, and other less clearly defined proteins (56–61). Studies of transgenic mice carrying chimeric hamster/mouse and human/mouse PrP genes have lead to the concept of "protein X," an intracellular component which would be required for the transconformation of PrP^{C} in the presence of PrP^{Sc} (3, 7, 62). However, a main difference between brain and lymphoid organs is that no significant pathological lesion occurs in the latter, while infection of the brain is always accompanied with vacuolar degeneration, astrocytosis, neuronal loss, and, in certain cases, amyloid deposition (63); thus, distinctive features differentiate the mechanisms of PrP^{Sc} processing and replication in neuronal versus lymphoid cells.

PrP^{Sc} could leave lymphoid organs and reach the central nervous system via nerve fibers or the blood stream. Both mechanisms may be hypothesized on the basis of existing data, and these possibilities are not mutually exclusive. Detection of the scrapie agent in organs such as lung and intestine 4 weeks after it appeared in lymphoid organs suggested that it spreads via the blood (29). Blood pools from several scrapie-infected hamsters were shown to contain infectivity from day 10 to day 40 post peripheral infection (64); however, no peak of infectivity could be detected preceding brain involvement (65). A leukocyte-enriched fraction (buffy coat) from guinea pigs infected with CJD was found to transmit the disease to recipient animals by intracerebral inoculation, and this property was persistent from the 1st to the 26th weeks after inoculation (66). Although we are not aware of any published data demonstrating infectivity of blood lymphocytes from diseased animals or patients, this possibility has been clearly raised (67). On the other hand, the sequential involvement of peripheral lymphoid organs, spinal cord, and then the brain argues in favor of a spreading through peripheral nerves (32). This possibility is also supported by the demonstration that very high doses of inoculum injected at the periphery are pathogenic in SCID (44) and RAG$^{0/0}$ (51) mice (Table 3), which lack B- and T-lymphocytes. Finally, lymphocyte fractions from spleens of scrapie mice appear to bear infectivity but not those from the blood; prions might either be fixed only on a noncirculating subset of splenic lymphocytes or bind to them only transiently (54).

Hence, the question of how cells supporting prion replication in the periphery deliver it to the nervous system remains unanswered. PrP^{Sc} replicates inside *in vitro* infected neuronal cells, but is not significantly released in the medium (68, 69). A likely hypothesis is that the spread of PrP^{Sc} mainly involves direct exchange between plasma membranes of adjacent cells, which would be facilitated by the glycosylphosphatidylinositol anchor (70). The routes of neuroinvasion

TABLE 2

Susceptibility to Scrapie in Mice Bearing Defined Immunodeficiencies, after Intracerebral and Intraperitoneal Inoculations (after Klein *et al.* (39))

Genotype	Defect	Intracerebral	Intraperitoneal	Remarks
$CD4^{0/0}$	CD4 T cells	+	+	
$CD8^{0/0}$	CD8 T cells	+	+	
$\beta 2m^{0/0}$	CD8 T cells	+	+	
Scid C57BL	T & B cells	+	6/8	Increased inc. time
Scid C.B-17	T & B cells	+	1/4	Increased inc. time
$RAG1^{0/0}$	T & B cells	+	0	
$RAG2^{0/0}$	T & B cells	+	0	
μMT	B cells	+	0	
t11μMT	antibodies	+	+	
$TNF\text{-}R1^{0/0}$	FDC	+	+	

might be peripheral nerves; however, lymphocytes, if they do not replicate prions themselves, appear to be required for the spreading of prions from cell to cell (probably to FDCs) within the lymphoid organs (54). Alternatively, it remains conceivable that very low amounts of prions, not detectable by infectivity tests, are continuously transported via the blood stream to the blood brain barrier. The particularly long incubation period of TSE may be explained by a very slow transport of prions to the central nervous system by blood cells or along the nerves or both.

CONCLUSIONS

Studies performed more than 20 years ago showed that factors known to act on the immune system could influence mouse susceptibility to scrapie infection. Mitogenic stimulation by phytohemagglutinin or bacterial lipopolysaccharide made mice susceptible to doses of prions that would have been otherwise ineffective and reduced the incubation time after peripheral inoculation by nearly 20% (43). On the other hand, administration of high doses of prednisone immediately before and after intraperitoneal inoculation of mice with a scrapie-infected brain homogenate resulted in prolonged incubation periods (42). Since these two early studies, no further publications have addressed the effects of immunomodulating agents on peripheral route prion infections. The major efforts at potential therapeutic approaches have focused on central nervous system events in clinically affected individuals, since the ability to identify patients at the preclinical stage of disease is limited. There is, however, a very long asymptomatic period, which provides a potential therapeutic window in individuals known to have been exposed. Since the recognition of BSE and vCJD, the potential population who is at an incubation stage from accidental exposure to prions may be increased. Methods for large-scale screening of potentially exposed or at-risk people exist (71) and will hopefully become more efficient and easy to perform. Hence, intervention at the preclinical stages is a promising goal. Prophylactic measures have already been empirically proposed, based on the results cited above (72), but they remain to be tested. Future studies on the mechanisms by which prions interact with the lymphoid cells will hopefully lead to a better design of drugs that could impair the peripheral steps of TSE.

TABLE 3

Transfer of Prion Susceptibility to $RAG1^{0/0}$ Mice by Fetal Liver Cells (after Klein *et al.* (51))

Donor	Dose of inoculum	Sensitivity to scrapie
C57BL/6	7–8 $logLD_{50}$	+
$Prnp^{0/0}$	7–8 $logLD_{50}$	+
$TCR\alpha^{0/0}$	7–8 $logLD_{50}$	+
μMT	7–8 $logLD_{50}$	+
C57BL/6	3–4 $logLD_{50}$	+
$Prnp^{0/0}$	3–4 $logLD_{50}$	+
$TCR\alpha^{0/0}$	3–4 $logLD_{50}$	+
μMT	3–4 $logLD_{50}$	0
None	6–7 $logLD_{50}$	0

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EXHIBIT C

Vaccination as a Therapeutic Approach to Alzheimer's Disease

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OUTLINE

ABSTRACT

Alzheimer's disease is the most common cause of dementia worldwide. Alzheimer's disease is a member of a broad range of neurodegenerative diseases characterized pathologically by the conformational change of a normal protein into a pathological conformer with a high β-sheet content that renders it neurotoxic. In the case of Alzheimer's disease, the normal soluble amyloid β peptide is converted into oligomeric/fibrillar amyloid β. The oligomeric forms of amyloid β have been hypothesized to be the most toxic, whereas fibrillar amyloid β becomes deposited as amyloid plaques and congophilic angiopathy, which both serve as neuropathological markers of the disease. In addition, the accumulation of abnormally phosphorylated tau as soluble toxic oligomers and as neurofibrillary tangles is a critical part of the pathology. Numerous therapeutic interventions are under investigation to prevent and treat Alzheimer's disease. Among the most exciting and advanced of these approaches is vaccination. Immunomodulation is being tried for a range of neurodegenerative disorders, with great success being reported in most model animal trials; however, the much more limited human data have shown more modest clinical success so far, with encephalitis occurring in a minority of patients treated with active immunization. The immunomodulatory approaches for neurodegenerative diseases involve targeting a self-protein, albeit in an abnormal conformation; hence, effective enhanced clearance of the disease-associated conformer has to be balanced with the potential risk of stimulating excessive toxic inflammation within the central nervous system. The design of future immunomodulatory approaches that are more focused is dependent on addressing a number of questions, including when is the best time to start immunization, what are the most appropriate targets for vaccination, and is amyloid central to the pathogenesis of Alzheimer's disease or is it critical to target tau-related pathology also. In this review, we discuss the past experience with vaccination for Alzheimer's disease and the development of possible future strategies that target both amyloid β–related and tau-related pathologies. ***Mt Sinai J Med 77:17–31, 2010.*** *© 2010 Mount Sinai School of Medicine*

Key Words: Alzheimer's disease, amyloid β, immunomodulation, tau, transgenic mice, vaccination.

Alzheimer's disease (AD), affecting approximately 37 million people currently, is the most common cause of dementia worldwide. In the United States, AD is the sixth leading cause of death, with an estimated 5.3 million Americans having AD. By 2050, according to some estimates, 1 of every 85 persons worldwide will be affected by AD.[1] Currently available treatments for AD provide largely symptomatic relief with only minor effects on the course of the disease. There is an urgent need for better therapeutic interventions. Besides

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immunomodulation, numerous other approaches are being studied, including anti–amyloid β (anti-Aβ) aggregation agents, secretase inhibitors/modulators blocking Aβ production, tau aggregation blockers, agents targeting mitochondria, stem cell therapies, and various neuroprotective strategies.[2] Perhaps the greatest hope for an intervention that shall significantly affect disease progression in the near future comes from the vaccination approaches.[3,4] Certainly in AD transgenic (Tg) mouse models, Aβ-directed immunization has been spectacularly successful with a wide variety of methods. However, significant unanswered questions remain for current and future human trials about the best design of a vaccine, the best target, and the timing of therapy initiation. A key issue that needs to be addressed is the targeting of both Aβ-related and tau-related pathologies.

When the best design for an AD vaccine is being determined, a key issue is the targeting of both Aβ-related and tau-related pathologies.

PATHOGENESIS OF FAMILIAL AND SPORADIC ALZHEIMER'S DISEASE

The pathological hallmarks of AD are the accumulation of Aβ as neuritic plaques and congophilic angiopathy and the accumulation of abnormally phosphorylated tau in the form of neurofibrillary tangles (NFTs). Missense mutations in amyloid precursor protein (APP) or in the presenilin genes [presenilin 1 (PRES1) and PRES2] can cause early-onset, familial forms of AD [familial Alzheimer's disease (FAD)] affecting <4% of AD patients. The most common form of AD is sporadic and of late onset. The dominant theory for the causation of AD has been the amyloid cascade hypothesis.[5,6] This theory currently suggests that the accumulation of Aβ peptides, particularly in a highly toxic oligomeric form, is the primary pathogenic driver that downstream leads to tau hyperphosphorylation, NFT formation, and ultimately synaptic and neuronal loss. Extensive evidence supports this hypothesis in FAD patients and in models of FAD:

1. Inherited forms of AD linked with mutations in the APP gene or in the PRES1 or PRES2 genes are associated with changes in APP processing that favor overproduction of soluble amyloid β (sAβ) or the production of more aggregation prone forms of sAβ such as Aβ 1–42.[7]
2. Down's syndrome, in which there is an extra copy of the APP gene due to trisomy 21, is associated with AD-related pathology at a very early age.[8]
3. In Tg and other models of coexpressed Aβ and tau, Aβ oligomer formation precedes and accentuates tau-related pathology, and this is consistent with the hypothesis that NFT formation is downstream from Aβ aggregation.[9–11]
4. In Tg mouse models of mutant APP overexpression (in which there is no tau pathology), therapeutic prevention and/or the removal of Aβ is associated with cognitive benefits in experimental mice.[12–15]

Importantly, in Tg mouse models of both mutant APP and tau overexpression (with both amyloid-related and tau-related pathologies), prevention of Aβ pathology leads to amelioration of both cognitive deficits and tau-related pathology.[16–18] However, evidence proving that Aβ is central in the common late-onset sporadic form of AD is more limited:

1. A correlation has been shown between biochemically extracted Aβ peptides species from sporadic AD brains with cognitive decline.[19]
2. Isolated Aβ peptide dimers/oligomers from sporadic AD brains have been documented to impair synaptic structure and function.[20]
3. Aβ extracted from sporadic AD patients has been shown to induce amyloid deposits when injected into Tg mice.[21]

A significant problem for the amyloid cascade hypothesis comes from the autopsy data from the initial human active vaccination trial, which is further discussed later. Postmortem analysis was available for 9 subjects in the active immunization arm.[22] All these individuals showed some degree of plaque removal and a reduced Aβ load in comparison with comparable nonimmunized controls. Despite this, there were no differences between placebo and active immunization groups in terms of long-term survival outcome, time to severe dementia, and outcome measures such as the Alzheimer Disease Assessment Scale–Cognitive (ADAS-Cog), Mini Mental State Examination (MMSE), and Disability Assessment for Dementia. This may be related to immunization being begun too late in the disease process; alternatively, one can use these data to suggest that the amyloid cascade hypothesis is an oversimplification. A number of investigators have suggested alternative theories in which the accumulation of Aβ and tau hyperphosphorylation are dual pathways both downstream from a common upstream pathogenic deficit (which remains to be

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identified).[23–25] In such a scenario, it is essential for immunotherapy to address both of these pathologies to be highly effective. Hence, in this review, we summarize the preclinical and clinical data for both Aβ and phosphorylated tau reduction immunotherapeutic approaches.

A number of investigators have suggested [that] the accumulation of Aβ and tau hyperphosphorylation are dual pathways both downstream from a common upstream pathogenic deficit (which remains to be identified).

PAST HUMAN EXPERIENCE WITH ACTIVE IMMUNIZATION TARGETING AMYLOID BETA

Initial data supporting immunotherapy for AD showed that anti-Aβ antibodies could inhibit Aβ peptide fibrillization/oligomerization and prevent cell culture–based neurotoxicity.[26,27] This led to vaccination of AD Tg mice with Aβ 1–42 or Aβ homologous peptides coinjected with Freund's adjuvant, which demonstrated striking reductions in Aβ deposition and, as a result, elimination of behavioral impairments.[12–15,28,29] Similar effects on Aβ load and behavior have been demonstrated in AD Tg mice by peripheral injections of anti-Aβ monoclonal antibodies, and this indicates that the therapeutic effect of the vaccine is based primarily on eliciting a humoral response.[30,31] In the initial preclinical studies, no toxicity was evident in the treated mice; however, some investigators suggested that the use of nonfibrillogenic, nontoxic Aβ homologous peptides along with approaches that stimulate primarily humoral, T helper 2 (Th2) immunity, in contrast to a primary T helper 1 (Th1) cell–mediated response, might reduce potential toxicity.[32–34] The dramatic biological effect of vaccination in preclinical testing encouraged Elan/Wyeth in April 2000 to launch a randomized, multiple-dose, dose-escalation, double-blind phase I clinical trial with a vaccine designated as AN1792, which contained pre-aggregated Aβ 1–42 and QS21 as an adjuvant. This type of vaccine design was aimed to induce a strong cell-mediated immune response because QS21 is known to be a strong inducer of Th1 lymphocytes.[35] The initial trial was conducted in the United Kingdom and involved 80 patients with mild to moderate AD.[36] This trial was designed to assess the antigenicity and toxicity of multiple-dose immunization with the full-length Aβ 1–42 peptide with QS21. Fifty-three percent of patients developed an anti-Aβ humoral response. During the later stages of the phase I trial, the emulsifier polysorbate 80 was added, causing a greater shift from a Th2-biased response to a proinflammatory Th1 response.[37] In the subsequent phase IIa trial begun in October 2001, 372 patients were enrolled, with 300 receiving the aggregated Aβ 1–42 (AN1792) formulation with QS21 in polysorbate 80. This trial was prematurely terminated in January 2002 when 6% of vaccinated patients manifested symptoms of acute meningoencephalitis (18 of 298 subjects).[35,38,39] Autopsies performed on a limited number of trial patients suggested that striking Aβ clearance of parenchymal plaques had occurred that was similar to what had been reported in the animal studies, and this confirmed the validity of this approach for amyloid clearance in humans.[39–44] In these cases, extensive areas of cerebral cortex were devoid of plaques, with residual plaques having a "moth-eaten" appearance or persisting as "naked" dense cores. This amyloid clearance in most cases was in association with microglia that showed Aβ immunoreactivity, which suggested phagocytosis. Additional striking features were the persistence of amyloid in cerebral vessels and unaltered tau immunoreactive NFTs and neutrophil threads in regions of the cerebral cortex in which plaque clearing had apparently occurred in comparison with regions without clearing.[42–44] Hence, this initial vaccination approach did not address vascular amyloid–related or NFT-related pathology. Some cases also showed a deleterious T cell reaction surrounding some cerebral vessels, which was suggestive of an excessive Th1 immune response. It appeared that the immune reaction triggered by AN1792 was a double-edged sword: the benefits of a humoral response against Aβ were overshadowed in some individuals by a detrimental T cell–mediated inflammatory response.[39,45] The likely involvement of an excess cell-mediated response in mediating toxicity was supported by an analysis of peripheral blood mononuclear cells from trial patients, which were stimulated in vitro with the Aβ peptide, followed by quantification of cytokine secretions by enzyme-linked immunosorbent spot assay.[37] The cells of most responder trial patients mounted interleukin 2 and interferon-γ–positive responses indicative of a class II (CD4+) Th1-type response.[37] Not all patients who received AN1792 responded

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with antibody production. The majority mounted a humoral response and showed a modest but statistically significant cognitive benefit demonstrated as an improvement on some cognitive testing scales in comparison with the baseline and a slowed rate of disease progression in comparison with the patients who did not form antibodies.[36,46] The follow-up data from the Zurich cohort, a subset of the Elan/Wyeth trial,[46,47] indicated that the vaccination approach may be beneficial for human AD patients. In agreement with the findings in the Zurich cohort, immune responders with high antibody titers in the multicenter cohort scored significantly better in composite scores of memory functions in comparison with low responders and nonresponders or with the placebo group of patients.[37] However, it is striking that despite the apparent success in amyloid clearance indicated by the autopsy data, the clinical cognitive benefits were very modest when the active vaccination group was compared to the placebo group.[48] No difference between the antibody responders and the placebo group was found on the ADAS-Cog, Disability Assessment for Dementia, Clinical Dementia Rating scale, MMSE, or Clinical Global Impression of Change. It was only on a 9-item composite neuropsychological test battery that antibody responders had a slight benefit in comparison with the placebo group. These data can be used to suggest that vaccination in this cohort was started too late; hence, tau-related pathology was unaffected by vaccination, and thus the cognitive benefits were small. Alternatively, it can be suggested that the amyloid cascade hypothesis must be an oversimplification of the pathogenesis of sporadic AD. The latter view is supported by a follow-up study of 80 patients in the initial phase I AN1782 trial, of whom 8 came to autopsy.[22] This study showed that despite evidence of very significant amyloid plaque removal in 6 of the 8 autopsy subjects, which correlated with the anti-Aβ titer, there was no evidence of improved survival or an improvement in the time to severe dementia in the overall group.[22]

PAST PASSIVE IMMUNIZATION EXPERIENCE FOR AD

Passive immunization consists of an injection of pre-prepared antibodies to patients, in contrast to active immunization, in which the immune system is stimulated to produce its own antibodies. Passive transfer of exogenous monoclonal anti-Aβ antibodies appears to be the easiest way to fulfill the goal of providing anti-Aβ antibodies without the risk of uncontrolled Th1-mediated autoimmunity. AD Tg model mice treated in this way had significantly reduced Aβ levels and demonstrated cognitive benefits.[30,31] Potential problems with passive immunization include the need for repeated injections in a chronic disease, the high cost, the proper selection of antigen targets, blood-brain barrier penetration, the risk of hemorrhages, and the development of an immune response to the injected antibodies. Several passive immunization trials are underway, with the most advanced being the phase III bapineuzumab trial begun in December 2007.[4] The phase II trial using this anti-Aβ monoclonal antibody was a randomized, double-blind, placebo-controlled trial testing 3 doses in 240 participants. In each of the escalating doses of the antibody, approximately 32 subjects received the active agent, and 28 received placebos. Although the study did not attain statistical significance for the primary efficacy endpoint in the whole study population, in the subgroup of non–apolipoprotein E4 carriers, clinically significant benefits were documented with a number of scales, including the MMSE and Alzheimer's Disease Assessment Scale Battery, over the 18-month trial period. In, addition among non–apolipoprotein E4 carriers, the evaluation of the magnetic resonance imaging results showed less loss of brain volume in treated patients versus control patients. However, it was reported that some patients in the treatment group developed vasogenic edema, a significant adverse reaction. The phase III trial is trying to recruit 800 patients and will run until December 2010.

With a somewhat similar approach, intravenous immunoglobulin (IVIg) is currently in clinical trial for AD, with the rationale being that IVIg contains some anti-Aβ antibodies. In a pilot, open-label study of 8 mild AD patients, IVIg was infused over 6 months, discontinued, and resumed for another 9 months.[49] After each infusion, the plasma Aβ levels increased transiently, with cerebrospinal fluid Aβ being decreased after 6 months. The MMSE increased by an average of 2.5 after 6 months, returned to the baseline after washout, and remained stable with the subsequent IVIg infusions. These promising initial findings clearly need to be repeated in a larger cohort. The attraction of IVIg use is that there is extensive experience using IVIg safely for multiple neurological disorders; however, it is a very expensive treatment, and the percentage of anti-Aβ antibodies in IVIg is extremely low, so this is not likely to be a very specific or highly effective form of treatment.

A particular concern in association with passive immunization is cerebral microhemorrhage. The mechanism of this hemorrhage is thought to be related to Aβ deposition in the form of

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congophilic amyloid angiopathy (CAA), which causes degeneration of smooth muscle cells and weakening of the blood vessel wall. A number of reports have shown an increase in microhemorrhages in different AD mouse models after passive intraperitoneal immunization with different monoclonal antibodies with a high affinity for Aβ plaques and CAA.[50–52] Microhemorrhages following active immunization in animal models have also been reported but only in 2 studies, so this appears to be less of a problem with this approach.[53,54] In Tg mouse models, Aβ antibodies can both prevent the deposition of vascular amyloid and remove it, thus contributing to vascular repair. On the other hand, the autopsies from the AN1792 trial indicated no clearance of vascular amyloid, and in one of these cases, numerous cortical bleeds were found, which are typically rare in AD patients.[41] This is an important issue as CAA is present in virtually all AD cases, with approximately 20% of AD patients having severe CAA.[55] Furthermore, CAA is present in about 33% of cognitively normal elderly control populations.[56] The need for vascular repair and regeneration during Aβ immunotherapy represents another argument for early treatment as well as an argument favoring subtle clearance over a longer time period.

PHOSPHORYLATED TAU AS AN IMMUNE TARGET

NFTs are a major pathological hallmark of AD. NFTs are intraneuronal inclusion bodies that consist of an accumulation of paired helical filaments (PHFs), which biochemically are mainly composed of abnormally phosphorylated tau. Recently, there has been increasing focus on phosphorylated tau as an immunotherapeutic target.[57–59] In the central nervous system, human tau is expressed in 6 isoforms arising from alternative messenger RNA splicing from a single gene on chromosome 17q21 containing 16 exons (see Figure 1).[60,61] The size range of

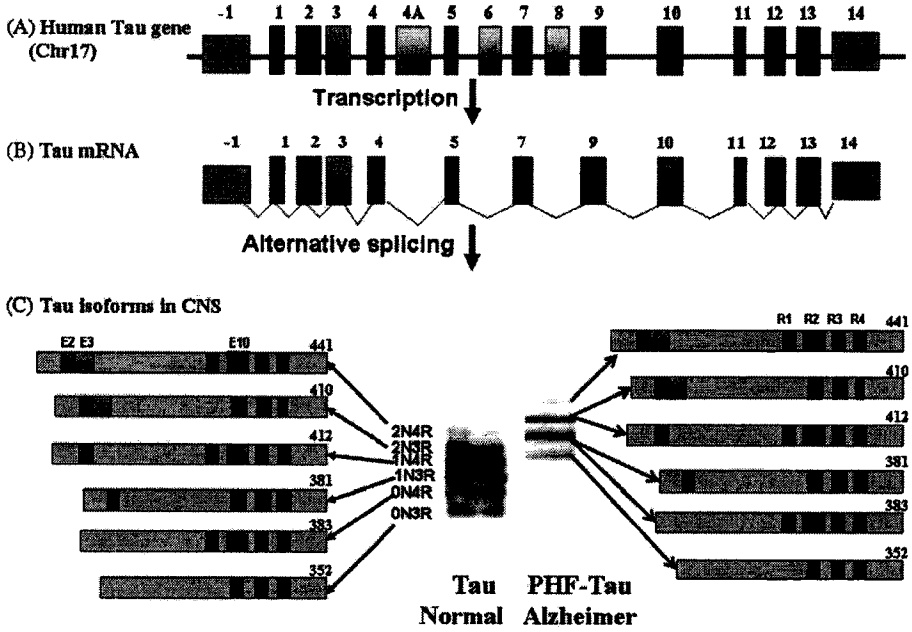

Fig 1. Schematic representation of the human tau gene, which is located on chromosome 17q21 and spans more than 130 kb. This gene is composed of 16 exons. (A) Exons 1 and 14 are transcribed but not translated (turquoise). Exons 4A, 6, and 8 are not transcribed in humans (light blue/charcoal). (B) In the human brain, 6 tau isoforms, ranging from 352 to 441 amino acids, are generated by alternative splicing of exons 2, 3, and 10 (brown/red, pink, and red, respectively) from a single gene. Exons 1, 4, 5, 7, 9, 11, 12, and 13 (blue) are included in all isoforms. Exon 3 is always included with exon 2. The microtubule binding domains are indicated by R1, R2, R3, and R4, which correspond to exons 9, 10, 11, and 12, respectively. (C) The extraction of tau proteins and PHF tau from normal and Alzheimer brains, respectively, shows via immunoblotting 6 bands between 45 and 68 kDa that correspond to different tau isoforms in the normal brain, whereas in PHF tau, 4 bands are detected between 60 and 74 kDa that correspond to the aggregation of 6 hyperphosphorylated tau isoforms in the Alzheimer brain. **Abbreviations:** CNS, central nervous system; mRNA, messenger RNA; PHF, paired helical filament.

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the 6 isoforms is 352 to 441 amino acids, which differ by the absence or presence of 29 (exon 2) or 58 (exon 2 + exon 3) amino acid inserts in the amino terminal. The carboxyl terminal half of tau contains 3 or 4 semihomologous repeats of 31 or 32 amino acids encoded by exon 10. The repeats (3R and 4R) correspond to the microtubule binding region of the tau protein (see Figure 1). Stabilization of microtubules by tau is essential for the maintenance of neuronal cell morphology and transport of organelles. In addition, tau has other roles such as interactions with kinesin 1 and the complex dynactin/dynein.[62,63] Tau also plays a crucial role in neuronal cell architecture by interacting with plasma membrane or cytoskeleton proteins such as actin, spectrin, and neurofilament proteins. Several mutations have been described in the tau gene in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and other tauopathies; however no tau gene mutations have been linked to the presence of AD.[64] Most of these mutations affect the binding of tau to microtubules or enhance the aggregation of tau into fibrils. Other intronic mutations that affect the splicing of exon 10 induce an increase in isoforms with 4 repeats. In AD, tau is hyperphosphorylated at all phosphorylated sites, with 9 phosphates per molecule, in comparison with normal brain tau, which has 2 to 3 phosphorylated residues.[65] Other studies have suggested that changes in tau splice forms are related to neurodegeneration. In some animal models expressing mutated tau, there is an increase in 4R tau versus 3R tau.[66] The functional significance of a shift in the 3R tau/4R tau ratio remains unclear, but 4R tau binds microtubules with a higher affinity than 3R tau.[67]

Normal tau and PHF tau differ in the molecular weight and banding pattern, as shown in Figure 1. Normal tau has 6 bands between 45 and 68 kDa, whereas PHF tau has 4 bands between 60 and 74 kDa (see Figure 1).[68,69] The diversity of tau isoforms is related to various posttranslational modifications such as phosphorylation, glycosylation, glycation, ubiquitination, and nitration.[70] The splicing regulation of the tau gene and the relative expression of isoforms are not significantly changed in sporadic AD (Figure 2).[71] Tau has multiple potential phosphorylation sites, which can be detected using specific phosphorylated tau-dependent antibodies (see Figure 3). Seventy-one of the 85 potential phosphorylated sites have been shown to be phosphorylated under physiological or pathological conditions.[72,73] More than 20 protein kinases have been implicated in the phosphorylation of tau proteins, with glycogen synthase kinase-3β and cyclin-independent kinase 5 thought to play the most important roles in phosphorylation under pathological conditions.[72-75]

Several Tg mouse models that express human tau with the FTDP-17 mutation have been produced (see Table 1). Some of these mice display NFTs, neuronal death, and behavioral deficits,[76-85] except for a Tg mouse model that expresses a mutated (N279K) tau that shows behavioral deficits without the formation of NFTs or neuronal loss.[86] In these models, there is disruption of axon transport due to the tau expression, which induces synaptic and neuronal loss. Another Tg tau mouse model was developed that expresses the mutated P301S tau, which shows synaptic loss that precedes tangles formation.[84] The distribution of NFTs in most of these tauopathy models is in contrast to AD because NFTs

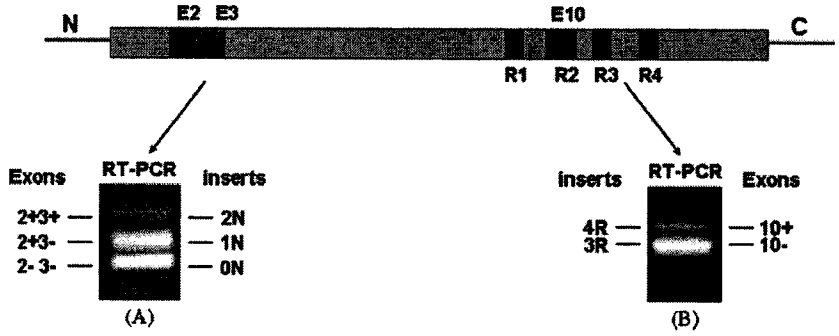

Fig 2. Electrophoresis of RT-PCR amplification products of (A) the 5′ domain of tau mRNAs and (B) the 3′ domain of tau mRNAs. The extraction of RNA was performed from the cerebellar cortex of a sporadic Alzheimer's disease patient. The expression of mRNA by RT-PCR showed different isoforms of human tau detected in the N-terminal (0N, 1N, and 2N) and in the C-terminal (3R and 4R). The plus symbol indicates with an exon, whereas the minus symbol indicates without an exon. **Abbreviations:** mRNA, messenger RNA; RT-PCR, reverse-transcriptase polymerase chain reaction.

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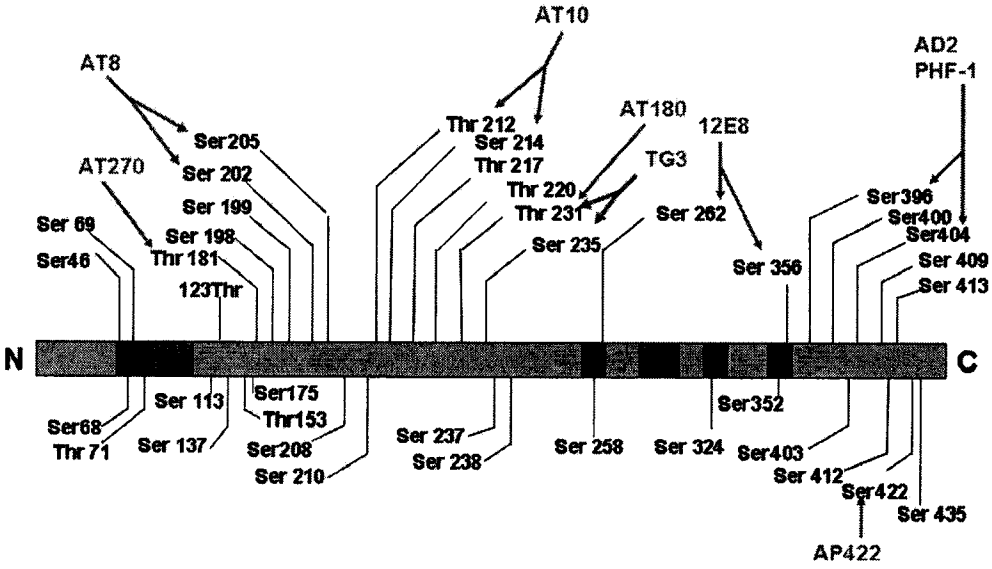

Fig 3. In the human Alzheimer brain, more than 40 phosphorylation sites on tau have been identified and localized in the proline-rich domain and in the C-terminal region. Phosphorylated sites are identified with 8 phosphorylated tau–specific antibodies, as indicated in Figure 1C, with red. It has been suggested that the phosphorylation at Ser 262/356 is responsible for the detachment of tau from microtubules. **Abbreviation:** PHF-1, paired helical filament 1.

are localized in different brain regions such as the brain stem, spinal cord, and frontotemporal cortex instead of the entorhinal region, hippocampus, and neocortex, as observed in AD.[87] In order to generate a more ideal model for AD, other researchers have used a single wild-type human tau to generate a Tg model; however, most of these models did not develop NFT, with the except of 2 models: one expressing ON3R wild-type tau with a few NFTs in aged animals[80] and another with abundant NFTs expressing all 6 human tau isoforms on a knockout background for murine tau.[88,89] The absence of tangles in mice that expressed a single wild-type human tau was likely due to the endogenous tau inhibiting the formation of an NFT-like pathology.

Recently, it has been shown that active immunization of Tg mice (P301L) with a phosphorylated tau peptide (containing the phosphorylated PHF-1 epitopes Ser 396 and Ser 404) for 2 to 5 months could prevent tau-related pathology.[90,91]

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These particular phosphorylation epitopes were chosen because these sites have been shown to increase the fibrillogenic nature of tau and contribute to PHF formation.[92,93] Histological and biochemical analyses showed a reduction of aggregated tau in the brain and improved performance on motor tasks.[90] This study clearly documented that it is possible to reduce tau-related pathology with active immunization.

At first examination, it is difficult to understand how an antibody response to a protein that is accumulating intracellularly can have beneficial effects. However, such an outcome is supported by a study of immunization in a Parkinson's disease Tg mouse model with α-synuclein showing a reduction of intracellular α-synuclein aggregates.[94] An additional study has shown that antibodies against Aβ can be internalized in AD neuronal culture models of Aβ accumulation and clear intraneuronal Aβ aggregates via the endosomal-lysosomal pathway.[95] Furthermore, recent evidence has shown that extracellular tau aggregates can be internalized and promote the fibrillization of intracellular full-length tau in a tissue culture model[96] and that the injection of fibrillar tau brain extract into the brains of Tg wild-type expressing mice can induce the formation of human tau into filaments as well as the spread of pathology from the site of injection into neighboring brain regions.[97] This type of infectivity of an abnormal protein conformation from outside the cell has also been demonstrated for polyglutamine aggregates[98] and is well characterized in prion disease.[99,100] Hence, if the spread of PHF

Table 1. *Different Transgenic Models Used to Study Tau-Related Pathology Expressing WT Tau or Mutated Tau Identified in Various FTDP-17 Pedigrees.*

Tau Isoform	Tau Mutation	Presenilin cDNA	APP cDNA	Promoter	NFT	Reference
2N4R	WT	–	–	Thy-1	No	150
2N4R	WT	–	–	Thy-1.2	No	151
2N4R	WT	–	–	Thy-1.2	No	152
0N3R	WT	–	–	HMG-CoAR	No	153
0N3R	WT	–	–	PrP	No	154
3R	WT	–	–	Mouse tubulin Tα1	No	155
0N3R	WT	PS1 M146L	–	HMG-CoAR	No	156
0N3R	WT	PS1 M146L	APP 751 (SL)	HMG-CoAR and Thy-1	No	157
2N4R	WT/KoKI	–	–	Thy-1	No	158
6 human isoforms	WT	–	–		Yes	88
0N4R	P301L	–	–	PrP	Yes	159
0N4R	P301L	–	APPsw	PrP	Yes	79
0N4R	P301S	–		Thy-1.2	Yes	160
0N4R	P301L	PS1 M146V	APPsw	Thy-1.2	Yes	10
2N4R	P301L	–	–	Thy-1.2	Yes	9
2N4R	G272V	–	–	PrP	Yes	161
2N4R	M337V	–	–	PDGF	Yes	81
2N4R	R406W	–	–	CaMKII	Yes	162
2N4R	R406W P301L G272V	–	–	Thy-1	Yes	163
2N4R	P301L	–	–	Thy-1	Yes	158
1N2R	G272V P301S			Thy-1.2	Yes	164

Abbreviations: APP, amyloid precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; cDNA, complementary DNA; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; HMG-CoAR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; NFT, neurofibrillary tangle; PDGF, platelet-derived growth factor; PrP, prion protein; PS1, presenilin 1; WT, wild type.

pathology in AD occurs via a prion-like mechanism, anti–phosphorylated tau antibodies would not need to enter cells in order to be effective.

QUESTIONS TO ADDRESS FOR A NEW GENERATION OF AD VACCINES

An initial question that needs to be addressed is when to begin a vaccination protocol. Extensive neuropathological data have established that by the time the earliest clinical signs of AD emerge, Aβ deposition may be close to reaching its peak and that NFT formation and neuronal loss are substantial but have not yet reached peak levels.[101,102] This would suggest that amyloid-directed therapy would need to begin very early, perhaps even before the mild cognitive impairment (MCI) stage, in order to have a maximal effect. This is consistent with the albeit limited autopsy data from the initial AN1792 study, which showed that despite evidence of very significant amyloid plaque removal in 6 of the 8 autopsy subjects, which correlated with the anti-Aβ titer, in the overall group, there was no evidence of improved survival or an improvement in the time to severe dementia.[22] Hence, there is a need for the identification of markers predicting the conversion from normal aging to very mild dementia/MCI. These include cerebrospinal fluid biomarkers such as.[103] Early fluorodeoxyglucose positron emission tomography changes in hippocampal glucose metabolism can predict the conversion of normal cognition to pathologically verified AD.[104] Studies in AD Tg models, using magnetic resonance imaging, suggest that paramagnetic amyloid binding ligands have potential for early amyloid detection and subsequent treatment effects.[105,106] However, direct imaging of

Extensive neuropathological data suggest that amyloid-directed therapy would need to begin very early, perhaps even before the mild cognitive impairment (MCI) stage, in order to have a maximal effect.

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amyloid deposits with agents such as Pittsburgh compound B using positron emission tomography is currently the most promising method for the identification of early amyloid deposits and for the identification of patients who will likely convert to MCI from normal aging and from MCI to early AD.[107,108] An alternative approach is to immunize by targeting both Aβ deposition and tau-related pathology. Such an approach has a higher probability of having a clearer effect on the clinical course, even if started when clinical symptoms are evident. Furthermore, if, as discussed previously, tau pathology is not downstream from amyloid deposits but represents a parallel pathology related to a common upstream cause, it will be essential to target tau-related pathology, regardless of how early vaccination treatment is initiated.

An alternative approach is to immunize by targeting both Aβ deposition and tau-related pathology.

Another significant issue that needs to be addressed in future studies is the development of better models for preclinical testing of vaccination approaches. There are many shortcomings to current Tg models of AD pathology. These include the fact that Tg amyloid deposits typically lack the extensive posttranslational modifications of AD amyloid and thus are much more soluble; this presumably allows them to be cleared more easily.[109] The rodent immune system is quite different from the human immune system, and this leads to significant differences in the toxic responses to amyloid deposition.[110] Most current tau Tg models reflect FTDP-related pathology in contrast to AD tau pathology, as discussed previously.[87] Relatively few of the vaccination approaches being developed have been tested in nonhuman primates or other non-Tg models of AD. These may represent more accurate models of the type of immune response that might be elicited in aged humans and better reflect the combination of true human Aβ-related and tau-related pathologies.[111–113] These models include rhesus monkeys, vervet monkeys, mouse lemurs, and aged beagles.[113–118] It is striking that in a recent 22-month active immunization trial of aged beagles, despite an approximately 80% reduction in cortical Aβ immunoreactivity, little cognitive improvement on multiple measures of learning and memory could be detected.[119] However, improvements in some executive functions were found, mirroring the modest improvements seen only in the z score of the Neurological Test Battery among patients in the AN1782 trial. These results reinforce the need to begin immunomodulation very early in the disease progression with a focus on preventing Aβ deposition rather than clearance of preexisting lesions as well as the likely need to target tau-related pathology concurrently.

Active vaccination approaches under development are aiming to avoid the excessive Th1 stimulation that resulted in encephalitis in a proportion of the AN1792 patients. Concurrently, the formulation of any active vaccine also has to overcome the problem of immunosenescence in the target patient population. One promising approach taken by several investigators is to alter the sequence of the Aβ peptide immunogen in order to remove or alter the major Th1 stimulator sites in the carboxyl terminus and the middle portion of Aβ, while focusing on the major Th2 stimulator site in the amino terminus.[28,33,120–122] These Aβ homologous peptide immunogens can be combined with various costimulator epitopes. An example of this approach is a combination with a synthetic, unnatural pan human leukocyte antigen DR–binding epitope (PADRE)[122] or a linkage to viral-like particles[123–125] to induce a primarily humoral immune response. These can be further combined with other immunostimulator carriers. For example, the Aβ Th2 amino terminal epitope can be combined with PADRE and macrophage-derived chemokine in a DNA epitope vaccine to drive robust Th2 responses.[126] The choice of adjuvant is also an important consideration. The use of polysorbate 80, a strong Th1 stimulating adjuvant, in the AN1792 trial is one factor likely contributing to the encephalitis in a minority of patients. The use of adjuvants such as alum that drive primarily a Th2 response is preferable.[29,119] The route of immunization also plays an important role. Stimulating mucosal immunity by nasal, gut, or transcutaneous vaccination has been shown to drive strong Th2 responses.[127–129] An alternative, nonmutually exclusive approach to enhancing vaccine design is to stimulate innate immunity and enable microglia/macrophages to phagocytose amyloid deposits. Over 20 years ago, Wisniewski *et al.*[130] noted that although brain-resident macrophages are unable to phagocytose amyloid, brain-infiltrating macrophages are plaque-competent. A number of recent studies suggest that only a small percentage of plaques are associated with peripheral origin macrophages and that these are required for plaque clearance.[131–133] Vaccination approaches based on this knowledge are now being developed. Stimulation of peripheral macrophages to enter the central nervous system and phagocytose amyloid has been achieved by stimulation of

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Toll-like receptor 9 with CpG,[134,135] via blockade of the CD40/CD40L interaction,[136] and by blockade of the transforming growth factor β–Smad2/3 innate signaling pathway.[137] These innate immunity stimulatory approaches can be used alone or in combination with adaptive immunity stimulation. Stimulating the innate immune system has the added potential advantage that it could be effective against both Aβ-related and tau-related pathologies.

Another important issue for future vaccination approaches is the best target for either active or passive immunization. Abundant evidence both in vivo and in vitro suggests that the most toxic species of Aβ are oligomers or Aβ-derived diffusible ligands,[138,139] with a similar line of evidence suggesting that tau oligomers are the most toxic form of phosphorylated tau.[59,84] Active vaccination or the use of monoclonal antibodies that specifically target Aβ oligomers, tau oligomers, or preferably both would be an ideal way to block AD-related toxicity.

Active vaccination or the use of monoclonal antibodies that specifically target Aβ oligomers, tau oligomers, or preferably both would be an ideal way to block AD-related toxicity.

A small number of preclinical studies targeting Aβ oligomers suggest that this methodology is potentially powerful and in need of further development.[140–144] An additional important factor to consider is that emerging evidence suggests that monomeric Aβ peptides have normal physiological functions in the brain such as neuroprotection and modulation of long-term potentiation,[145,146] with phosphorylated tau also having a normal role.[58] Targeting only oligomeric Aβ or tau would avoid the potential of interfering with these physiological functions. A novel immunotherapeutic approach is to target the shared abnormal β-sheet conformation of amyloid proteins with conformationally specific antibodies or active immunization that favors such a conformational response.[140,141,147] Such an approach has the advantage that both Aβ-related and tau-related pathologies would be addressed concurrently.

CONCLUSION

Numerous therapeutic approaches are under development for AD; however, harnessing the immune system to clear both Aβ-related and tau-related pathologies is perhaps the most promising and advanced approach. Abnormal protein conformation is thought to be the underlying pathogenesis of not only AD but also a long list of neurodegenerative conditions, such as prion disease, Parkinson's disease, and Huntington's chorea, with immunomodulation having the potential to be a disease-altering therapeutic approach for all these disorders. For example, it has been shown that prion-directed mucosal vaccination can prevent infection from an exogenous source.[148,149] Ultimately, directing the immune system to clear the highly toxic, abnormal oligomeric conformers that characterize multiple neurodegenerative diseases has the potential to dramatically alter the course of a wide spectrum of age-associated diseases.

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DISCLOSURES

Potential conflict of interest: Nothing to report.

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EXHIBIT D

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Papillomavirus Pseudovirus: a Novel Vaccine To Induce Mucosal and Systemic Cytotoxic T-Lymphocyte Responses

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Intestinal mucosa is a portal for many infectious pathogens. Systemic immunization, in general, does not induce a cytotoxic T-lymphocyte (CTL) response at the mucosal surface. Because papillomavirus (PV) naturally infects mucosa and skin, we determined whether PV pseudovirus, i.e., PV-like particles in which unrelated DNA plasmids are packaged, could generate specific mucosal immunity. We found that the pseudovirus that encoded the lymphocytic choriomeningitis virus gp33 epitope induced a stronger CTL response than a DNA vaccine (plasmid) encoding the same epitope given systemically. The virus-like particles that were used to make the pseudoviruses provided an adjuvant effect for induction of CTLs by the DNA vaccine. The PV pseudovirus pseudoinfected mucosal and systemic lymphoid tissues when administered orally. Oral immunization with the pseudovirus encoding human PV type 16 mutant E7 induced mucosal and systemic CTL responses. In comparison, a DNA vaccine encoding E7, when given orally, did not induce a CTL response in intestinal mucosal lymphoid tissue. Further, oral immunization with the human PV pseudovirus encoding E7 protected mice against mucosal challenge with an E7-expressing bovine PV pseudovirus. Thus, PV pseudovirus can be used as a novel vaccine to induce mucosal and systemic CTL responses.

The mucosal surfaces of the body are readily infected with many pathogenic viruses and bacteria. In particular, the intestinal mucosa is an important portal for infectious agents. Most pathogens initiate their infectious processes by interaction with epithelial cells at mucosal surfaces and then spread systemically. To prevent initial infections by those pathogens, antibodies and cytotoxic T lymphocytes (CTLs) specific for the pathogens induced at the mucosal surface are of great importance. Because some pathogens continue to replicate in the mucosa, it is advantageous to induce mucosa-specific CTLs to clear the pathogens at initial infection and during the early stage of disease.

Intestinal mucosal lymphoid cells are located in organized lymphoid tissue, such as Peyer's patches, or in diffuse lymphoid tissue, such as lamina propria. Peyer's patches are considered the site where a mucosal immune response is induced after a pathogen invades the mucosa (24). In general, systemic immunization, such as subcutaneous vaccination, does not effectively induce mucosal immune responses; instead, mucosal immunization is required to generate an intestinal mucosal immune response.

DNA (plasmid)-based immunization induces host humoral and cellular immune responses (1, 3, 5, 12, 13, 31, 39, 43). Because antigens encoded by plasmid DNA vaccines are produced in the host, the antigens retain their natural form, unlike those of attenuated whole-organism vaccines, which are denatured and modified. Because the antigens are expressed in the immunized host, there is prolonged exposure to the host immune system and sustained immune responses. However, DNA vaccines do not reach gut-associated lymphoid tissues via oral immunization because they do not survive degradation in the gastric and intestinal environment. Furthermore, DNA vaccines induced relatively low amounts of CTLs and generated CTLs in some but not all immunized individuals when given intramuscularly to mice and humans (4, 29, 34, 38, 51).

Papillomaviruses (PVs) are a group of small DNA viruses that naturally infect skin and mucosal surfaces (52). More than 95 types have been characterized so far (45). PV major protein L1 can be assembled spontaneously into virus-like particles (VLPs) when expressed in insect cells, yeasts, and even bacteria (10, 15, 27, 35, 37, 46). It has been shown that PV VLPs can induce strong humoral and cellular immune responses when used for systemic immunization (6, 10, 11, 18, 20, 33, 37, 40, 44, 49, 50). Further, VLPs can be used to package unrelated plasmids to form PV pseudoviruses (14, 42). Because many PVs are mucosatropic and can induce cellular immune responses, we hypothesized that PV pseudoviruses would reach the mucosal immune system and induce mucosal immune responses. Because PV VLPs were shown to induce strong T-helper responses, we hypothesized that the concurrent T-helper responses to the VLPs might enhance the CTL response against the antigen encoded by the plasmid in the pseudoviruses. In this study, we found that when administered orally, PV pseudoviruses reached Peyer's patches, lamina propria, and spleen. By systemic immunization, PV pseudoviruses induced a stronger CTL response than plasmid DNA vaccines alone, and by oral immunization, they generated specific mucosal and systemic CTL responses and protected mice against mucosal challenge.

MATERIALS AND METHODS

Cells. RMA, RMA-neo, and RMA-E7 cells were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

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Plasmids. Plasmid pCI-neo was purchased from Promega (Madison, Wis.). The expression cassette for the green lantern protein (GLP) was constructed by inserting the full-length GLP cDNA into the *Not*I site of plasmid pCI-neo. The expression cassette for a fusion protein, GLP fused with lymphocytic choriomeningitis virus (LCMV) gp33 major histocompatibility complex (MHC) class I H-2D^b-restricted epitope (amino acids [aa] 33 to 41; KAVYNFATC), was constructed by using PCR with pCI-GLP as the template, oligonucleotide 5′ primer GCCACCATGAGCAAGGGCGAGGAACTGT, and 3′ primer TCAACAGG TGGCAAAATTGTAGACAGCCTTAGATCCGCCGCCACCGCCACCCTT GTACAGCTCGTCCAT, containing the linker sequences (Gly_6Ser_1; underlined) between GLP and LCMV gp33 epitope. The amplification mixtures (50 μl) contained dGTP, dATP, dTTP, dCTP (200 μM each), oligonucleotide primers (1 μM), template DNA (25 ng), and *Taq* DNA polymerase (Promega) (5 μM). The reaction mixture was subjected to 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final 10 min at 72°C. The amplified DNAs were gel purified and ligated into T-easy vector (Promega). Then the DNAs were digested with *Eco*RI and ligated into pCI-neo, which had been digested with the corresponding enzyme. The human PV type 16 (HPV-16) E7 open reading frame was fused to the GLP sequence by PCR using the same linker (Gly_6Ser_1). The fragment was then inserted into pCI-neo to form pCI-GLP-E7. The E7 open reading frame was inserted into pCMV.

Generation of recombinant baculoviruses. Briefly, *Spodoptera frugiperda* (Sf9) cells were grown in monolayer cultures at 27°C in TNMFH medium (Sigma, St. Louis, Mo.) supplemented with 10% FCS and 2 mM glutamine. Ten micrograms of transfer plasmid (pVL1933 BPV-1 L1Δ or pVL1932 HPV-16 L1Δ) was used to transfect Sf9 cells together with 0.2 μg of linearized Baculo-Gold DNA (Pharmingen, San Diego, Calif.). Recombinant viruses were purified using methods modified as described previously (26, 28).

Purification of PV VLPs. Sf9 cells were grown to a density of 1×10^6 to 2×10^6 cells/ml in TNMFH medium supplemented with 10% FCS and 2 mM glutamine in a spinner flask. Approximately 2×10^8 cells were pelleted at 1,500 × *g* for 5 min, resuspended in 10 ml of medium, and then added to 10 ml of recombinant baculoviruses at a multiplicity of infection of 2 to 5 for 1 h at room temperature. After addition of 125 ml of medium, the cells were plated on five round dishes (150 mm in diameter) and incubated for 3 to 4 days at 27°C. Cells were harvested, pelleted, and suspended in 10 ml of extraction buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 150 mM NaCl, 20 mM HEPES, 0.01% Triton X-100). The cells were sonicated for 1 min at speed 3; then the extract was pelleted at 10,000 rpm in a Sorvall RC5B centrifuge at 4°C for 30 min. The pellet was suspended in 8 ml of extraction buffer, sonicated again for 30 s at speed 4.5, and centrifuged again. Combined supernatants were layered on a two-step gradient with 14 ml of 40% sucrose on top of 8 ml of CsCl solution (4.6 g of CsCl per 8 ml of extraction buffer) and centrifuged in a Sorvall AH629 swinging-bucket rotor for 2 h at 27,000 rpm at 10°C. The interphase between CsCl and sucrose and the complete layer of CsCl were collected and placed in 13.4-ml Quickseal tubes filled with extraction buffer. Samples were centrifuged overnight at 50,000 rpm at 20°C. Gradients were fractionated by puncturing tubes on top and bottom with a 21-gauge needle, and 5 μl of each fraction was analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Western blot analysis. The extracts from infected insert cells were separated by SDS–10% PAGE and transferred to nitrocellulose by using a semidry blotting system (Semi Dry blotting unit; Fisher Biotech, Hanover Park, Ill.). The membranes were blocked overnight with 5% nonfat dry milk and incubated with mouse anti-HPV-16 L1 monoclonal antibody (Pharmingen) or rabbit anti–bovine PV type 1 (BPV-1) L1 antibody. Then the membranes were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG. Finally, the membranes were processed with the ECL system (Amersham, Arlington Heights, Ill.). Positive fractions were tested for the presence of VLPs by electron microscopy.

Production of PV pseudoviruses. Disassembly and reassembly of the recombinant HPV-16 VLPs and BPV-1 VLPs were done according to a modification of the procedure of Touze and Coursaget (42). Briefly, 5 μg of purified HPV-16 VLPs or BPV-1 VLPs (theoretically 1.5×10^{11} particles) was incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 10 mM EGTA, and 20 mM dithiothreitol (DTT) in a final volume of 100 μl at room temperature for 30 min. At this step, 1 μg of expression plasmid in 50 mM Tris-HCl buffer and 150 mM NaCl were added to the disrupted VLPs. The preparation was then diluted with $CaCl_2$ (25 mM) and 20% dimethyl sulfoxide in equal volume at room temperature for 1 h. The preparations were treated with 10 U of Benzonase (Bz) with or without proteinase K (pK; 1 mg/ml) for 1 h at room temperature, and the presence of plasmid DNA was determined by agarose gel electrophoresis to verify whether the DNA plasmid was packaged into the VLPs. Additionally, 0.5 μg of plasmid DNA (about 7×10^{10} copies of plasmid) was incorporated into 200 μl of pseudoviruses.

Electron microscopy. Twenty microliters of each fraction from CsCl gradients was dialyzed against 10 mM HEPES for 45 min on floating filter pads (0.02-μm pore size; Millipore, Bedford, Mass.). Carbon-coated copper grids (200 mesh size; EM Sciences, Gibbstown, N.J.) were treated with 20 μl of poly-L-lysine (1 mg/ml; Sigma) for 2 min. The sample was placed onto the grid for 2 min. Spotted grids were then stained with 30 μl of uranyl acetate solution for 2 min. Excess stain was removed, and grids were air dried. Specimens were examined with a Zeiss EM 900 electron microscope.

Mice. Six- to eight-week-old female C57BL/6 mice (purchased from the Jackson Laboratory, Bar Harbor, Maine, or Harlan, Indianapolis, Ind.) were used. All mice were kept under pathogen-free conditions. The protocol was approved by the Institutional Animal Care and Use Committees.

Immunization. For systemic immunization, mice were immunized subcutaneously with 100 μl of HPV pseudoviruses (about 3.5×10^{10} pseudoviruses or 0.25 μg of plasmid), 100 μl of HPV VLPs, 20 μg of plasmid in 100 μl of phosphate-buffered saline (PBS), or 100 μg of peptide (LCMV glycoprotein [gp] aa 33 to 41) in 100 μl of incomplete Freund's adjuvant (IFA). On day 14 after immunization, each group of five mice was given a booster of 100 μl of BPV pseudoviruses (about 3.5×10^{10} pseudoviruses or 0.25 μg of plasmid), 100 μl of BPV VLPs, 20 μg of plasmid, or 100 μg of peptide (LCMV gp aa 33 to 41) in IFA. For mucosal immunization, mice were immunized orally by gavage with 100 μl of PV pseudovirus, 100 μl of VLPs, or 20 μg of plasmids in 100 μl of PBS as a negative control and boosted in the same way on day 14.

Detection of systemic CTLs. Two weeks after the booster immunization, mice (five per group) were sacrificed, and spleen cells were isolated from each mouse. After incubation in nylon wool columns for 1 h at 37°C and 5% CO_2, enriched T cells were washed through the column with complete cell culture medium (RPMI 1640 medium, including 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). Cells were cultured at 37°C and 5% CO_2 for 7 days in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 U of interleukin 2 (IL-2) per ml, and 5 μg of E7 peptide aa 49 to 57 (RAHYNIVTF, H-2D^b-restricted epitope) per ml, or the LCMV gp peptide. Specific cytolytic activity was determined by a ^{51}Cr release assay (see below).

Isolation of Peyer's patches and MLN cells. Briefly, after mice were sacrificed, mesenteric lymph nodes (MLN) were removed from the mesenteric tissue, and Peyer's patches were identified and removed from small intestine. Single-cell suspensions were prepared in complete cell culture medium. Because freshly isolated mucosal T cells undergo apoptosis in vitro, their specific cytolytic activity was determined immediately by ^{51}Cr release assay.

In vitro cytotoxicity assay. Target cells (10^6 RMA-E7, RMA-neo, or RMA cells) were labeled with ^{51}Cr (100 μCi) for 1 h at 37°C and washed three times. The RMA cells were further loaded with peptides by directly adding peptides to the cells at 5 μg/ml. Target cells (2,000 cells per well) were then incubated with effector cells at different effector/target ratios in V-bottomed 96-well microtiter plates for 6 h at 37°C. Supernatant was collected, and ^{51}Cr release was quantified by γ counter (ICN Biomedical Inc., Huntsville, Ala.). Specific lysis was calculated according to the formula [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release was determined in control microcultures containing ^{51}Cr-labeled target cells in culture medium with no effector cells. Maximum release was determined by lysing ^{51}Cr-labeled target cells with 0.5% (vol/vol) NP-40.

ELISPOT assay for IFN-γ-secreting cells. The enzyme-linked immunospot (ELISPOT) assay described by Taguchi et al. (41) was modified to detect specific CD8 T lymphocytes. First, 96-well filtration plates (Millipore) were coated with rat anti-mouse gamma interferon (IFN-γ) antibody (Pharmingen). Threefold dilutions of spleen cells in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, 2-mercaptoethanol, and antibiotics were added to the wells along with 10^5 γ-irradiated (50 Gy) feeder spleen cells and 10 U of recombinant human IL-2 (Pharmingen) per well. Cells were incubated for 48 h with peptide stimulation. After culture, the plates were washed followed by incubation with biotinylated anti-mouse IFN-γ antibody (Pharmingen). Spots were developed using freshly prepared substrate buffer (0.33 mg of 3-amino-9-ethyl-carbazole per ml and 0.015% H_2O_2 in 0.1 M sodium acetate, pH 5).

Confocal microscopy. The tissue slides (~5 to 7 μm) were fixed in cold PBS containing 2% formaldehyde for 10 min and examined with a Zeiss EM 900 confocal microscope. Images were captured and recorded with software provided by Zeiss.

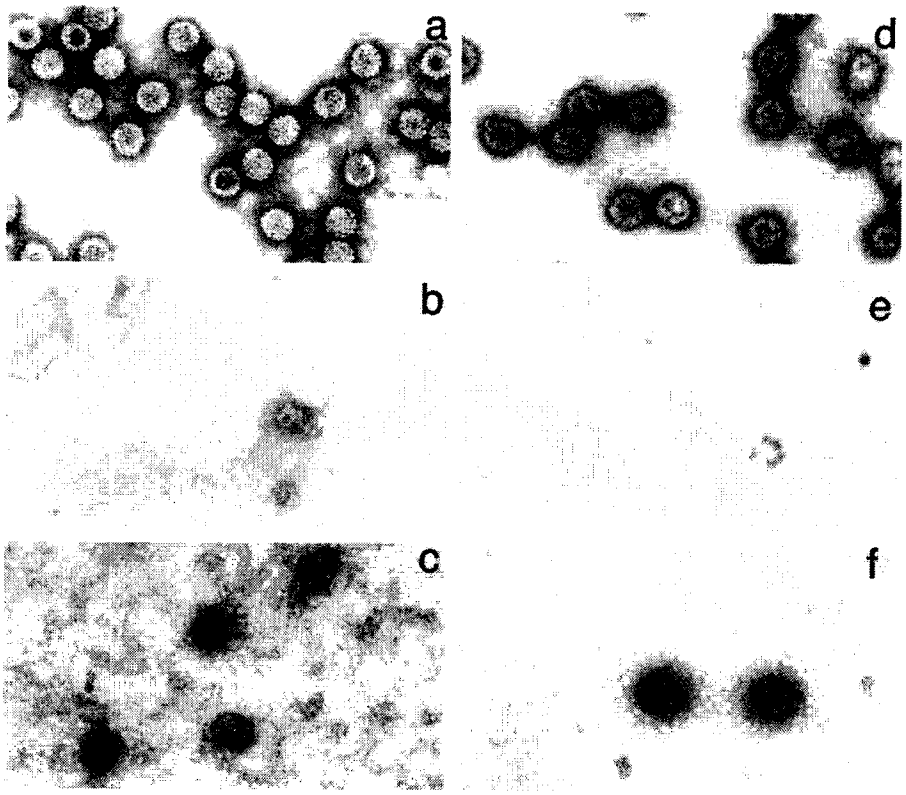

FIG. 1. Electron micrographs of VLPs derived from BPV-1 L1 or HPV-16 L1, EGTA- and DTT-disrupted VLPs, and pseudoviruses. The VLPs were disrupted with EGTA and DTT; then the plasmid pCI-GLP was added. The VLPs were refolded by adding increasing concentrations of $CaCl_2$ to form PV pseudoviruses. (a) BPV VLPs; (b) disrupted BPV VLPs; (c) BPV pseudoviruses; (d) HPV VLPs; (e) disrupted HPV VLPs; (f) HPV pseudoviruses. Magnification, ×84,000.

Statistical analysis. The differences among groups were compared by analysis of variance. Between-group comparisons were made with the Duncan test. A two-sided alpha level of 0.05 was considered statistically significant.

RESULTS

Production of PV pseudoviruses. HPV-16 L1 and BPV-1 L1 VLPs were produced in Sf9 cells using recombinant baculoviruses (26, 28). Briefly, the cells were infected by recombinant baculoviruses encoding either HPV-16 L1 or BPV-1 L1 with a C-terminus deletion. The C-terminus deletion has been shown to enhance production of PV VLPs (28). Three days after infection, the cells were lysed and VLPs were purified on CsCl and sucrose gradients. Gradients were fractionated (1 ml per fraction); then 5 μl of each fraction was analyzed by SDS–10% PAGE and Western blotting. The fractions positive for the L1 protein were examined for the presence of VLPs by electron microscopy. The fractions containing VLPs were dialyzed for 1 h against 10 mM HEPES (pH 7.5). BPV-1 and HPV-16 VLPs (Fig. 1a and d) were added to an equal volume of buffer containing EGTA and DTT and then incubated at room temperature for 30 min. Under these conditions, VLPs were completely disrupted (Fig. 1b and e). Plasmid DNA (pCI-GLP) was then added, and the preparation was incubated with $CaCl_2$ and dimethyl sulfoxide in order to refold VLPs (Fig. 1c and f). Most of the L1 proteins seemed to reassemble into VLPs under those conditions. To determine whether the plasmid DNA was packaged in the VLPs or on their surfaces, Bz was used after the refolding to digest DNA on the surfaces of the VLPs. Then the pseudovirions were treated with pK so that the VLPs were disrupted, and the presence of plasmid DNA inside the VLPs was determined by agarose gel electrophoresis. We found DNA plasmid in Bz- and pK-treated pseudovirus, indicating that the plasmid DNA was packaged in the VLPs (Fig. 2).

PV pseudoviruses induced a stronger CTL response than a DNA vaccine. To test whether PV pseudoviruses induce a stronger CTL response than a plasmid DNA vaccine, we immunized mice subcutaneously with an HPV-16 pseudovirus

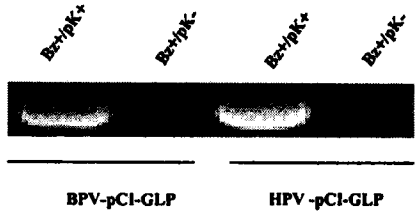

FIG. 2. Encapsidation of plasmid pCI-GLP DNA by PV VLPs. The pseudoviruses were treated with Bz to digest the DNA on the surfaces of VLPs and with pK to verify whether the plasmid DNA was packaged inside the VLPs and then subjected to electrophoresis. Initially, 1 μg of the plasmid DNA was used to make the pseudovirus, and after digestion of 200 μl of pseudovirus with Bz and pK, 0.5 μg of the plasmid remained.

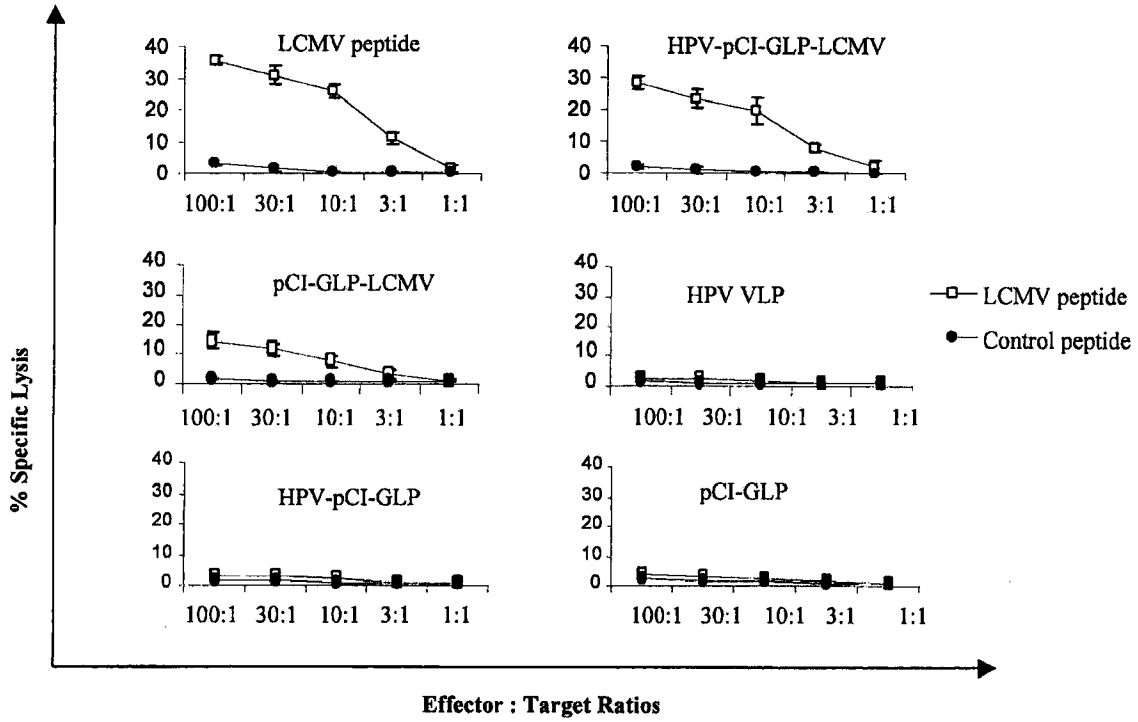

FIG. 3. PV pseudovirus induced a stronger LCMV-specific CTL response than a DNA vaccine. Mice were subcutaneously immunized with PV pseudovirus with a plasmid encoding the GLP-LCMV gp33 epitope (aa 33 to 41) fusion protein or GLP, with VLPs alone, with the plasmid alone (pCI-GLP-LCMV or pCI-GLP), or with the LCMV gp peptide (aa 33 to 41) in IFA. A ^{51}Cr release assay was used to measure the LCMV gp33 epitope-specific CTLs in spleen lymphocytes. The target cells (RMA) were pulsed with LCMV peptide (aa 33 to 41) or with a control peptide (HPV-16 E7 aa 49 to 57). The data are means ± standard deviations (five mice per group).

containing a plasmid encoding the H-2D^b-restricted epitope (9) of LCMV gp (aa 33 to 41) fused to GLP sequences (HPV-pCI-GLP-LCMV) or the plasmid alone (pCI-GLP-LCMV). pCI-GLP, HPV-16 VLPs, and the pseudovirus encoding GLP (HPV-pCI-GLP) were used as negative controls, and LCMV gp peptide (aa 33 to 41) in IFA was the positive control. Fourteen days after immunization, mice were given subcutaneous boosters of BPV-1 pseudovirus encoding GLP-LCMV or GLP, the plasmid pCI-GLP-LCMV or pCI-GLP, the BPV-1 VLPs, or the gp33 peptide. Fourteen days after the booster, spleen cells were isolated and then incubated with LCMV gp33 peptide (aa 33 to 41) in T-Stim culture supplement (Collaborative Biomedical Products, Bedford, Mass.) (without concanavalin A) in 5% CO_2 at 37°C for 1 week. Standard ^{51}Cr release assay was used to detect LCMV-specific CTLs using murine RMA cells loaded with LCMV peptide or control peptide (HPV-16 E7 aa 49 to 57) as target cells. We found that PV pseudoviruses encoding the LCMV epitope induced a stronger CTL response than the plasmid encoding the LCMV epitope (Fig. 3). Furthermore, by using the ELISPOT assay, we found that PV pseudoviruses generated three times more IFN-γ-producing CD8$^+$ cells specific for the LCMV peptide than the plasmid alone (Table 1).

PV VLPs serve as an adjuvant for a DNA vaccine to induce CTL response. To test whether VLPs had an adjuvant effect on CTL induction by the DNA vaccine, we immunized mice (five per group) with the plasmids alone (20 μg), with the plasmids (20 μg) plus BPV VLPs (2.5 μg), or with VLPs alone (2.5 μg) as a control and then measured the generation of LCMV gp33-specific CTLs by IFN-γ ELISPOT. In the group immunized with the plasmids alone, 10.7 ± 7.25 (mean ± standard deviation) LCMV gp33-specific CTLs per 2 × 10^4 spleen cells were generated. In contrast, 23.35 ± 1.26 gp33-specific CTLs per 2 × 10^4 spleen cells were induced in mice immunized with the plasmids plus the VLPs. VLPs alone did not induce specific T cells. Thus, coimmunization with the plasmids and VLPs induced significantly more CTLs than immunization with the

TABLE 1. PV pseudovirus induced more specific IFN-γ-producing CD8$^+$ T cells than a DNA vaccine[a]

Immunization	No. of spots per 2 × 10^4 spleen lymphocytes
LCMV gp peptide (aa 33–41) + IFA	45.6 ± 2.09
PV-pCI-GLP-LCMV (pseudovirus)	23.0 ± 1.13
pCI-GLP-LCMV	7.9 ± 2.7
VLPs	0
PV-pCI-GLP (pseudovirus)	0
pCI-GLP	0

[a] Mice were immunized with HPV pseudovirus containing a plasmid encoding GLP fused to LCMV gp33 epitope (aa 33 to 41) or GLP, with VLPs alone, with the plasmid pCI-GLP-LCMV, pCI-GLP alone, or LCMV peptide plus IFA (aa 33 to 41) and then given boosters of BPV pseudoviruses, VLPs alone, or plasmids alone. Two weeks after the boosters, spleen lymphocytes pooled for five mice were incubated in plates that were coated with anti-IFN-γ antibody. Two days later, biotin anti-IFN-γ antibody was added and incubated overnight. Then streptavidin-horseradish peroxidase was added. The spots were counted after being developed by 3-amino-9-ethylcarbazole substrate. Data are means ± standard deviations for triplicates.

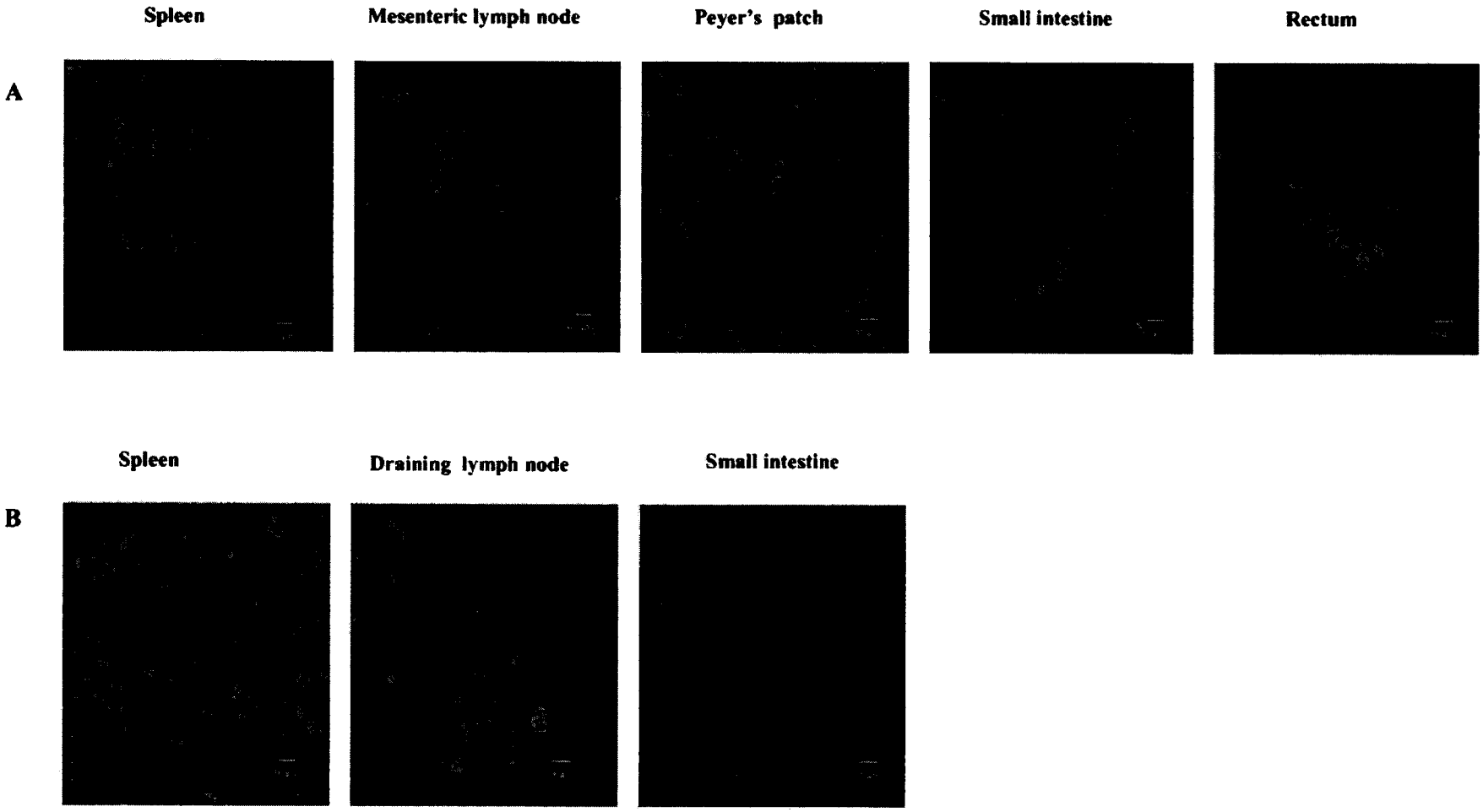

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FIG. 4. PV pseudovirus pseudoinfected intestinal mucosa and systemic lymphoid tissue when given orally. (A) HPV and BPV pseudoviruses encoding GLP were administered orally to mice. GLP expression was determined in the indicated tissues by confocal microscopy. GLP was found in the lamina propria of small and large intestines, Peyer's patches, MLN, rectum, and spleen but not in the muscles (data not shown). The data from mice given HPV pseudoviruses are shown. (B) BPV and HPV pseudoviruses encoding GLP were administered to mice by subcutaneous injection. GLP expression was found in draining lymph nodes and spleen but not in mucosa. The data from mice given BPV pseudoviruses are shown.

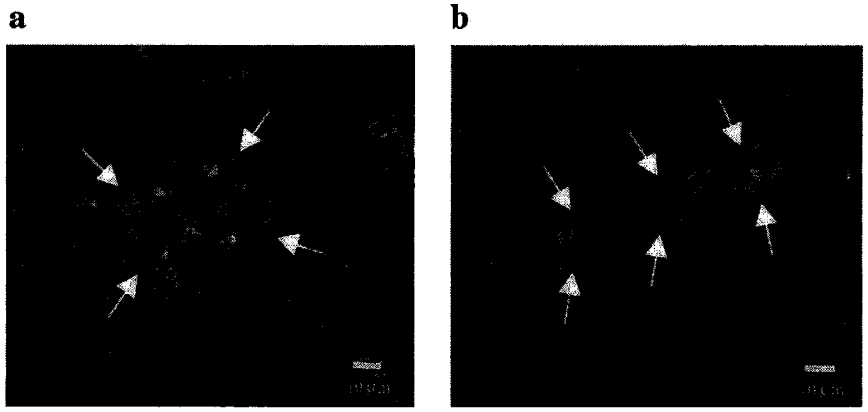

FIG. 5. Intestinal CD11b- and CD11c-positive cells colocalized with GLP expression in mucosal tissues of mice orally fed PV pseudoviruses encoding GLP. One day after oral administration with the pseudovirus encoding GLP, the mucosal tissues of the mice were removed and stained with anti-mouse CD11b or CD11c antibody. Phycoerythrin-labeled second antibodies were used after washing. GLP, CD11b, and CD11c expression was determined in the mucosal tissues by confocal microscopy. The expression of GLP (green), CD11c (red in panel a), and CD11b (red in panel b) in lamina propria (lamina propria is indicated by arrows) is shown.

plasmids alone ($P < 0.05$), indicating that the VLPs are an adjuvant for generating CTLs by the DNA vaccine.

PV pseudoviruses pseudoinfect mucosal and systemic lymphoid tissues. To test whether PV pseudoviruses pseudoinfect mucosal and systemic lymphoid tissues, we determined whether oral administration with the pseudovirus containing a plasmid encoding GLP (PV-pCI-GLP) resulted in expression of GLP in the mucosal and systemic lymphoid tissues. If the pseudoviruses pseudoinfected mucosal and systemic lymphoid tissues, we would be able to detect the expression of GLP by confocal microscopy. Thus, we fed mice (five per group) with HPV or BPV pseudoviruses (HPV-pCI-GLP or BPV-pCI-GLP), the VLPs alone (HPV VLPs or BPV VLPs), or the plasmid alone (pCI-GLP). At 1 and 7 days after feeding, mice were sacrificed; small intestines, rectums, spleens, MLN, and muscles were removed immediately and frozen. Tissue sections were made, and GLP expression was determined by confocal microscopy. We found expression of GLP in Peyer's patches, lamina propria, rectum, spleen, and MLN at day 1 (Fig. 4A) and at day 7 (data not shown). When the PV pseudoviruses were given subcutaneously, GLP expression was found in draining lymph nodes and spleen but not in the mucosal tissues (Fig. 4B). To determine which cells were infected by the pseudoviruses, we stained the tissues with phycoerythrin-labeled antibodies directed against CD11b, CD11c, CD3, and CD19 and determined whether they colocalized with GLP. Some of the $CD11b^+$ and $CD11c^+$ cells colocalized with the GLP (Fig. 5), suggesting that macrophages and dendritic cells were pseudoinfected with PV pseudoviruses. No $CD3^+$ or $CD19^+$ cells colocalized with the GLP.

Induction of specific CTLs in mucosal and systemic lymphoid tissues after oral immunization with PV pseudoviruses. To determine whether orally administered PV pseudoviruses induced mucosal and systemic CTL responses, we used a plasmid encoding an HPV-16 E7 mutant that has been shown to be highly effective in inducing CTL responses systemically (38). Mice (five per group) were fed by gavage with HPV-16 pseudovirus encoding the E7 mutant (HPV-pCMV-E7), a plasmid encoding the E7 mutant (pCMV-E7), or HPV-16 VLPs only. Fourteen days after feeding, they were given boosters of BPV-1 pseudovirus encoding the E7 mutant (BPV-pCMV-E7), the plasmid pCMV-E7 only, or BPV-1 VLPs only. Fourteen days after the booster, lymphocytes were isolated from MLN and Peyer's patches or spleen. The lymphocytes from MLN and Peyer's patches were used immediately to detect E7-specific CTLs. Spleen lymphocytes were stimulated with an E7 peptide (aa 49 to 57; RAHYNIVTF) for 1 week. A standard ^{51}Cr release assay was performed. We found that T cells from the mice immunized orally with PV-pCMV-E7 had mucosal and systemic CTL responses against E7-expressing target cells (Fig. 6). Oral immunization with the plasmid alone or PV VLPs did not induce an E7-specific CTL response. Pseudoviruses did not induce a mucosal immune response when mice were immunized by subcutaneous injection (data not shown).

Oral immunization with PV pseudovirus did not induce systemic tolerance. Because oral administration with soluble proteins might induce systemic tolerance (16, 22, 47, 48), we tested whether PV pseudoviruses induced systemic tolerance after oral immunization. We fed mice (five per group) with HPV-pCMV-E7, pCMV-E7, or HPV VLPs alone. Fourteen days after oral immunization, we immunized mice subcutaneously with BPV-pCMV-E7. Spleen T cells were isolated and then incubated with the E7 peptide, T-stim medium (without concanavalin A) in 5% CO_2 at 37°C for 1 week. A standard ^{51}Cr release assay was used to detect specific CTLs by using RMA-E7 and RMA-neo as target cells. All three groups of mice had CTLs against target RMA-E7 cells but not against RMA-neo cells (Fig. 7).

Oral immunization with PV pseudovirus provided immunity against mucosal challenge. To test whether oral immunization with the PV pseudoviruses induced mucosal protection, we immunized mice with HPV-16 pseudovirus encoding E7 and challenged them with BPV-1 pseudovirus encoding GLP-E7. We hypothesized that if HPV-16 pseudovirus induced a protective immune response, the mucosal immune system would clear BPV-1 pseudovirus-infected cells. Such a response would be indicated by an absence or decrease of GLP expression in

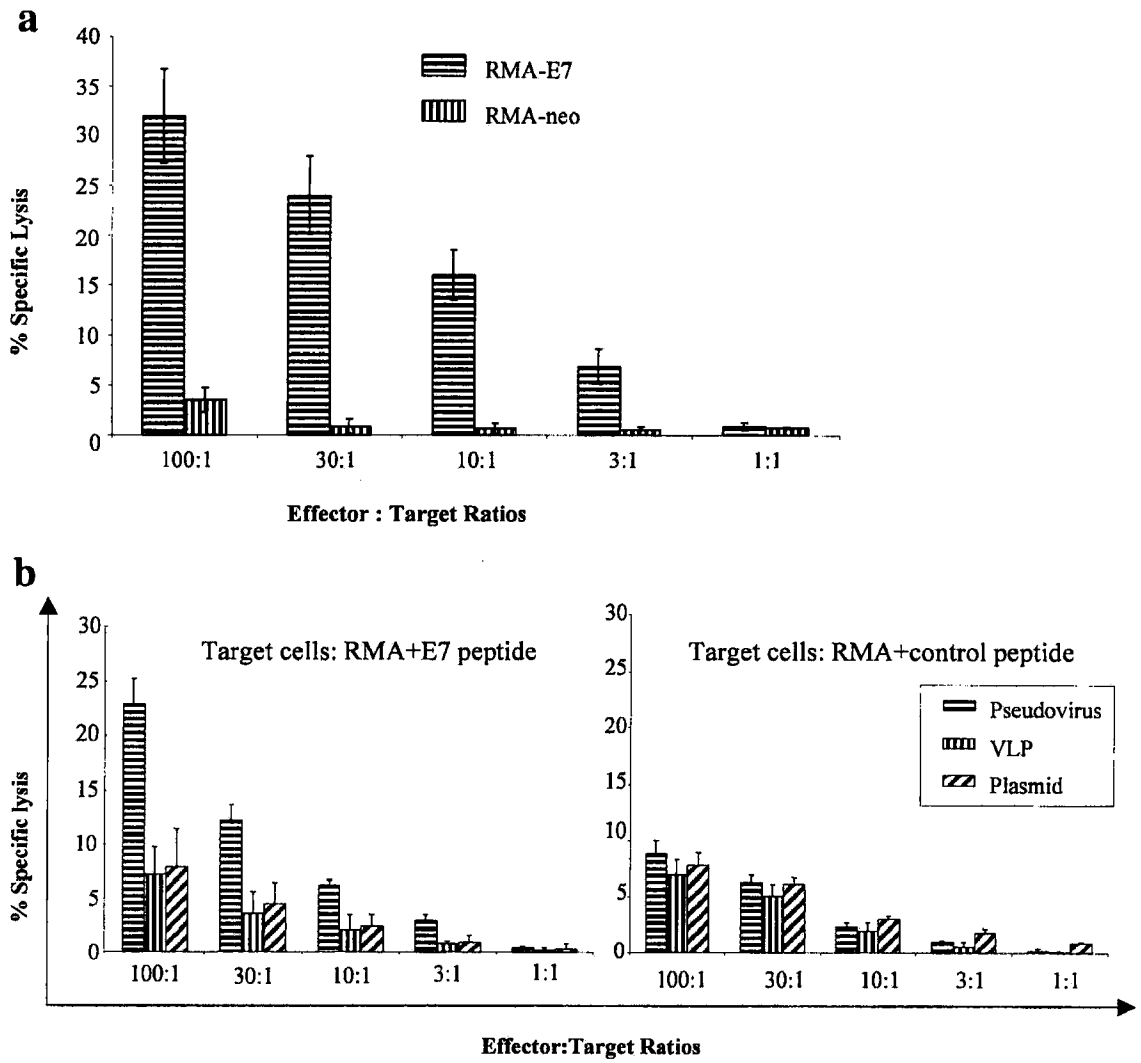

FIG. 6. Oral immunization with PV pseudovirus encoding E7 induced mucosal and systemic E7-specific CTLs. Mice were orally immunized with HPV-16 pseudovirus with a plasmid encoding a mutant E7, VLPs alone, or the plasmid alone and then given a booster of BPV pseudovirus, VLPs alone, or the plasmid alone. (a) Peyer's patches and MLN cells were isolated and immediately used to test E7-specific CTLs without in vitro restimulation. A ^{51}Cr release assay was used to measure E7-specific CTLs. The target cells were RMA-E7, which express the E7 antigen, and RMA-neo cells (negative controls). RMA-E7 and RMA-neo expressed comparable MHC class I levels (data not shown). The lymphoid cells from mice fed the plasmid or VLPs did not lyse the RMA-E7 cells (data not shown). (b) Spleen lymphocytes were isolated and restimulated with E7 peptides (aa 49 to 57) in vitro. The ^{51}Cr release assay was used to measure E7-specific CTLs. The target cells (RMA) were pulsed with an E7 peptide (aa 49 to 57) or with a control peptide. The data are means ± standard deviations for five mice per group.

the mucosal tissue of HPV-16 pseudovirus-immunized mice compared to the control-immunized group. To this end, we fed mice (five per group) with HPV-pCMV-E7, pCMV-E7, or HPV-16 VLPs. Fourteen days after oral immunization, the mice were given boosters of HPV-pCMV-E7, pCMV-E7 only, or HPV-16 VLPs. Fourteen days after the booster, all three groups of mice were challenged with BPV-1 pseudovirus encoding the GLP-E7 fusion protein. One day later, mice were sacrificed, and GLP expression in Peyer's patches was determined. The GLP expression was markedly lower in Peyer's patches of mice immunized with PV pseudovirus (4 ± 1 [mean ± standard deviation] green spots in each microscopic field) than in mice immunized with VLP (20 ± 3 green spots) or plasmid (21 ± 4 green spots) ($P < 0.05$) (Fig. 8).

DISCUSSION

One of the most important features of the PV pseudoviruses is their ability to reach mucosal and systemic lymphoid tissues. We showed that the plasmids themselves could not reach mucosal and systemic lymphoid tissues when administered orally, possibly because they did not survive degradation in the gastric and intestinal environment. Our data suggest that PV VLPs are resistant to the low-pH environment in the stomach and to proteolysis in the intestine.

Although we do not know which cells take up the pseudoviruses, it is likely that M cells in the follicle-associated epithelium have an important role in sampling the pseudoviruses and delivering them into the Peyer's patches. It is also possible that

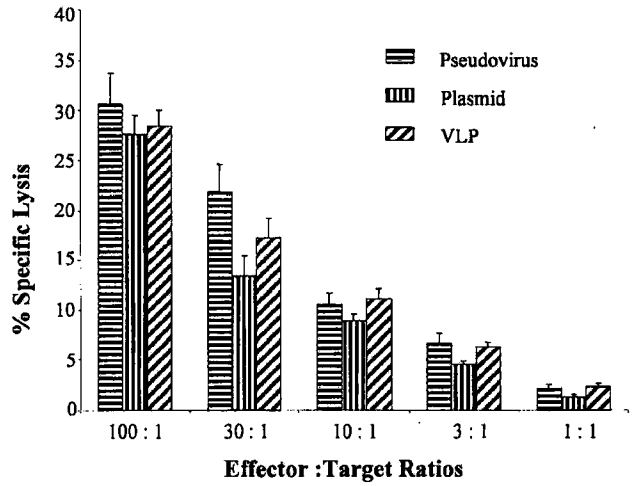

FIG. 7. Oral immunization with PV pseudovirus did not induce systemic tolerance. Mice were orally fed HPV-16 pseudovirus with a plasmid encoding HPV-16 E7, VLPs alone, or the plasmid alone; then mice were systemically immunized with BPV pseudovirus (PV) encoding the E7 protein. Spleen lymphocytes were isolated and restimulated with E7 peptides (aa 49 to 57) in vitro. A ^{51}Cr release assay was used to detect E7-specific CTLs. The target cells (RMA) were pulsed with an E7 peptide (aa 49 to 57) or with a control peptide (data not shown). The data are means ± standard deviations for five mice per group.

epithelial cells take up the pseudoviruses, because we observed GLP expression in lamina propria of the small intestine and rectum of mice fed PV pseudoviruses encoding GLP. PV VLPs have been shown to bind cells from different tissues and species (25, 32). We also found that PV pseudoviruses pseudoinfect dendritic cells and macrophages in lamina propria and Peyer's patches, which suggests that the pseudoviruses might cross the epithelial layers. It remains to be investigated how pseudoviruses pass through the epithelium and get into lamina propria dendritic cells and macrophages. We also found that the pseudoviruses reached MLN and spleen. It is possible that the dendritic cells and macrophages in lamina propria that had taken up the pseudoviruses moved to MLN and spleen directly. However, we cannot exclude the possibility that the pseudoviruses themselves directly reached MLN and spleen.

We also administered PV pseudoviruses encoding GLP to nostrils of mice and vaginal mucosae of rabbits and found that GLP was expressed in the mucosae of the respiratory tract and female reproductive tract (data not shown). It is thus likely that immunization with the pseudoviruses in these mucosal tissues might also generate CTL responses in respiratory tract and cervicovaginal mucosa. In fact, it has been shown that intranasal immunization with PV VLPs resulted in mucosal antibody responses (2, 19), suggesting that mucosal cellular immune responses can be induced at those sites.

Another important feature of the PV pseudoviruses is that they can induce a stronger CTL response than a DNA vaccine when administered systemically in mice. We have also shown that coinjection of PV VLPs with a plasmid DNA vaccine induced a stronger CTL response than immunization with the DNA vaccine alone. This demonstrates that PV VLPs actually serve as an adjuvant for the DNA plasmids to induce CTL responses. Because the VLPs that are used to package the plasmid DNA can induce VLP-specific T-helper responses, the T-helper cells might enhance the generation of CTLs specific for the antigen encoded by the plasmid through bystander action. Indeed, the VLPs can induce a strong Th1 response (7, 8, 17, 21); thus, it is likely that IL-2 produced by the Th1 cells amplifies the proliferation of CTLs. After the uptake of the pseudoviruses, antigen-presenting cells might be activated by the VLPs of the pseudoviruses, thereby expressing more costimulatory molecules, such as CD80 or CD86. Indeed, PV VLPs are shown to infect and activate human and murine dendritic cells (17, 36). The PV VLP-activated murine dendritic cells expressed an enhanced level of costimulatory molecules such as CD80 and produced proinflammatory cytokines, such as IL-6 and tumor necrosis factor alpha (17). These activated antigen-presenting cells could have an enhanced capacity to activate naïve CTLs specific for the antigen encoded by the plasmid compared with dendritic cells transduced by the plasmid (DNA vaccine) alone.

Oral immunization with PV pseudoviruses induced mucosal and systemic CTL responses. The intestinal mucosal CTLs

FIG. 8. Oral immunization with pseudovirus protected mice against mucosal challenge. Mice were orally immunized with an HPV-16 pseudovirus with a plasmid encoding E7, VLP alone, or the plasmid alone and then given boosters of the same agent 14 days later. On day 28, all three groups of mice were orally challenged with BPV pseudovirus encoding the GLP-E7 fusion protein. One day later, mice were sacrificed to detect GLP expression in Peyer's patches. The GLP expression was markedly lower in the Peyer's patches from the pseudovirus-immunized mice than in those from the VLP- and the plasmid-immunized mice.

were detectable among freshly isolated lymphocytes from Peyer's patches, suggesting that a significantly large number of specific CTLs were generated in Peyer's patches. Furthermore, oral immunization with the pseudoviruses protected mice against a mucosal pseudoviral challenge, strongly suggesting that the oral immunization with the pseudoviruses was protective. The protection is probably not a result of anti-HPV L1 VLP IgA, which cross-reacts with BPV pseudovirus and prevents its uptake or promotes its clearance, because there was no protection in mice immunized with HPV L1 VLPs alone. Further, the protection cannot be from E7-specific antibody responses, because E7 is a cytoplasmic protein (30) and the mutant E7 we used here was an unstable protein and was degraded intracellularly (38). Thus, protection is apparently mediated by CTLs specific for the E7 protein. The loss in GLP expression in the Peyer's patches was detected only 1 day after mucosal challenge in mice, which reflects the immediate antigen-specific CTL effector activity in mucosal tissues. Our data confirm those of a study that found that mucosal CTL responses are sustained once induced (23).

Because PV pseudoviruses pseudoinfect mucosal and systemic lymphoid tissues, they can be used as a gene delivery vector. After mice were fed with PV pseudoviruses encoding GLP, expression of GLP was found from the following day until week 3. Those data suggest that the expression of the gene delivered to the mucosal and systemic lymphoid tissues is transient. Thus, PV pseudoviruses can be used to deliver genes that are needed in the immune system for a short period. For example, they might be used to deliver immunomodulatory cytokine genes, such as the IL-10 gene to intestinal mucosa for Crohn's disease to suppress the Th1 type mucosal immune responses or the IL-12 gene to the upper respiratory tract to switch off the Th2 immune responses for asthma.

PV pseudoviruses are nonreplicating vectors. Their advantage over other live vectors is that they are composed of PV VLPs and plasmids, so there is no danger that they will revert to a virulent form. Although there are concerns about the integration of the DNA plasmids into the host genome, so far it has not been shown that the DNA plasmids cause any neoplasm. PV VLPs are made of L1 protein, which has not been shown to have detrimental effects on the host. The cells infected by the pseudoviruses, in general, will be deleted by specific CTLs because they induce CTL responses to the pseudoviruses. Although the pseudoviruses do not replicate in the host, the immunogen is expressed in the host antigen-presenting cells, leading to longer exposure of antigens to T cells.

In conclusion, PV pseudoviruses generated a stronger CTL response than a DNA vaccine and induced protective mucosal and systemic CTL responses; thus, PV pseudoviruses represent a novel vaccine for preventing and treating infections by pathogens at the mucosal surface.

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EXHIBIT E

Microbial Pathogenesis 1997; **22**: 219–225

MICROBIAL PATHOGENESIS

Cell mediated immunity induced in mice by HPV 16 L1 virus-like particles

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Recombinant human papillomavirus (HPV) type 16 L1 virus-like particles (VLPs) expressed in the baculovirus system were used to investigate the cellular immune response to human papillomavirus type 16. The cell-mediated immune response was evaluated through immunization of mice with HPV 16 L1 virus-like particles using a lymphoproliferation assay and cytokine production and cytometric analysis of lymphocyte subsets. A significant proliferative response was observed which was associated with secretion of both interferon-γ and interleukin-2. FACS analysis of splenic lymphocytes revealed that $CD8^+$ T-cells were increased in the immunized mice. These results demonstrate that HPV 16 L1 VLPs induce a T-cell response characterized by a Th1 profile and confirm that the HPV 16 VLP is a reasonable candidate for vaccine development.

Key words: HPV, cervical cancer, virus-like particles, cell mediated immunity, cytokines, Th1 profile.

Introduction

Human papillomavirus type 16 (HPV 16) infects genital epithelial cells and has been closely associated with the development of malignant lesions of the cervical squamous epithelium [1]. In order to prevent HPV infection and related diseases, immune response to these viruses during natural infection needs to be understood. To date little information has been reported regarding the immune response to HPV infection. The existence of a cellular immune response was evidenced in HPV infected patients who elicited a specific skin test reaction against recombinant HPV 16 L1 protein [2]. Cell mediated immunity may be essential in the control of HPV infection since HPV-associated malignancies occur more frequently in individuals with depressed cellular immunity [3, 4]. The difficulties of obtaining HPV by cell culture has led to the use of synthetic peptides [5–10] and recombinant HPV protein [11, 12] to analyse the immune response to HPV infection. The major capsid protein L1 of HPV 16 has recently been expressed in eukaryotic systems such as recombinant vaccinia virus and recombinant

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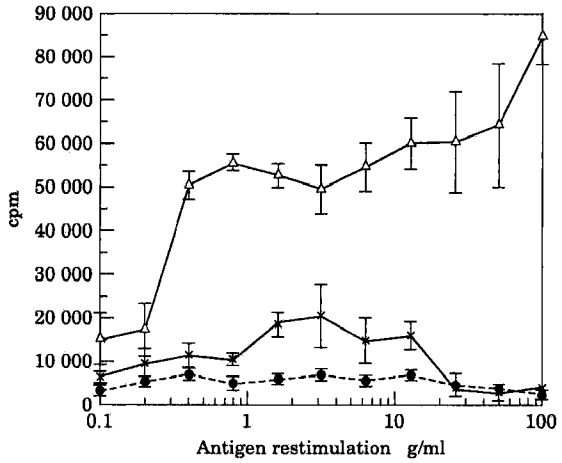

Figure 1. *In vitro* proliferation of primed lymphocytes restimulated with HPV 16 L1 VLPs, with the negative control antigen (WT) or with HBc VLPs. Results are the means of triplicate experiments ±SD and represent the combined results of three independant experiments. —△—: L1 16 VLPs; —×—: HBc VLPs; --●--: WT.

baculovirus [13]. Expressed L1 protein was shown to self-assemble into virus-like particles and thus may be of immunologic interest. A growing body of evidence indicates that HPV virus-like particles (VLPs) are the most valuable antigen to prevent HPV infections and related malignant lesions. Immunization with HPV 11 virions elicits neutralizing antibodies in animals and these antibodies can inhibit the infectivity of the virus in xenograft experiments [14–16]. These neutralizing antibodies recognize conformational epitopes of the viral capsid protein.

In a canine model, immunization with formalin-inactivated canine oral papillomavirus (COPV) protects dogs against COPV challenge [17, 18]. Immunization of cottontail rabbits with VLP's composed of the cottontail rabbit papillomavirus (CRPV) L1 major capsid protein expressed in the baculovirus expression system has recently been shown to protect rabbits against CRPV challenge [19–21].

In this study, we have investigated the cell mediated immune response in mice following immunization with recombinant HPV 16 capsids by means of lymphoproliferation assay (LPA), phenotype analysis of lymphocyte subsets and characterization of cytokine production.

Table 1. Splenic lymphocyte subsets in mice immunized with 5μg of HPV 16 VLPs and in control mice immunized with aluminium hydroxide alone.

	% of cells/spleen			
Mice	B	T	CD4	CD8
Control	24	11	25	9
Immunized	31	20	33	18

Results are representative of five mice treated independently of at least two separate experiments.

Results

Proliferative T-cell response to HPV 16 recombinant VLPs

Lymphocytes from primed HPV 16 VLP mice displayed a significant specific proliferative response (80 000 cpm) following *in vitro* incubation with HPV 16 VLPs whereas the WT antigen and the unrelated VLPs were unable to trigger a proliferative response of these lymphocytes. Moreover lymphocytes from naive mice did not proliferate whatever the stimulating antigen used.

Phenotype analysis of splenocytes after HPV 16 L1 VLP infection

To monitor the development of an immune response after immunization, spleen lymphocytes were counted and a phenotype analysis was performed.

The number of spleen lymphocytes from immunized mice with HPV 16 VLPs was significantly higher than that of the non-immunized group. Comparison of the B and T cell populations from each group revealed dramatic differences (Table 1).

The number of T-cells in immunized mice was significantly increased compared to the non-immunized mice ($P=0.0002$).

There was a significant difference in the number of B cells in both groups ($P=0.0002$). Analysis of T cell subpopulations showed a significant increase in the $CD8^+$ subset ($P=0.006$) and an obvious but not significant difference in the $CD4^+$ subset ($P=0.103$).

Table 2. Quantification of cytokines in culture supernatants of primed and unprimed lymphocytes.

		Cytokine release (pg/ml)			
Stimulator	Splenocytes	IFN-γ	IL-2	IL-4	IL-5
HPV 16 VLPs	Primed	6000	200	<1	<20
	Unprimed	200	11	<1	<20
ConA	Primed	250	494	10	5
	Unprimed	<5	490	12	<20
BSA	Primed	<5	8.4	<1	<20
	Unprimed	<5	8.2	<1	<20

Mice were immunized with 5 μg of HPV 16 VLPs adsorbed on 0.2% aluminium hydroxide.
Detection limits of the assays were respectively 5 pg/ml (IFN-γ), 2pg/ml (IL-2), 1pg/ml (IL-4) and 20 pg/ml (IL-5). The results represent the combined results of two experiments.

Table 3. Quantification (pg/ml) of cytokines in culture supernatants of primed lymphocytes and purified CD8 primed lymphocytes.

		Cell population		
Cytokine	Stimulator	Whole	$CD8^+$	$CD8^-$
IFN-γ	HPV 16 VLPs	2050	600	150
	HBc VLPs	100	80	150
IL-2	HPV 16 VLPs	310	63	360
	HBc VLPs	50	10	30

Mice were immunized with 5 μg of HPV 16VLPs adsorbed on 0.2% aluminium hydroxide or with 15 μg/ml of HBc VLPs. Detection limits of the assays are respectively 5 pg/ml (IFN-γ) and 2 pg/ml (IL-2)

Cytokine synthesis by HPV 16 L1 VLP stimulated lymphocytes

The supernatants from splenocyte culture were examined for the presence of IFN-γ, IL-2, IL-4 and IL-5 by ELISA (Table 2).

Significant IFN-γ production was measured after stimulation of primed splenocytes with HPV 16 L1 VLPs (6000 pg/ml), compared to only a small amount of IFN-γ produced by unprimed lymphocytes when stimulated by HPV 16 L1 VLPs (200 pg/ml). Increased IL-2 secretion was observed with HPV 16 L1 VLP T-cells (200 pg/ml) compared to unprimed T-cells (11 pg/ml). IL-4 and IL-5 were not detected in either group.

A further experiment was performed to characterize the lymphocyte populations involved in secretion of IL-2 and IFN-γ (Table 3). The results obtained show that most of the IFN-γ induced by primed lymphocytes was due to $CD8^+$ T-cells (2050 pg/ml) whereas primed $CD8^-$ T-cells seemed to be involved in IL-2 production (360 pg/ml). Moreover, the HBc VLPs produced with the same expression system as that used for HPV 16 were unable to stimulate secretion of both IL-2 and IFN-γ.

Discussion

In this study, we demonstrated that L1 capsids from HPV 16 produced in a recombinant baculovirus system were able to stimulate a significant T-cell response in immunized mice. The T-cell response observed was specific since insect cells and baculovirus components as well as unrelated VLPs produced on the same system did not stimulate the primed T-cells.

FACSort analysis revealed that the number of $CD4^+$ and $CD8^+$ T-cells was greatly increased after immunization with L1 capsids. The ratio of CD4/CD8 lymphocytes from immunized mice was depressed compared to control mice showing a significant enlargement of the $CD8^+$ T-cell subset. These results are consistent with those describing the reversion of the CD4:CD8 ratio in the tissue and peripheral blood of humans with condylomata acuminatum and the recruitment of $CD8^+$ T-cells to the site of neoplasia in the cervix [22–24].

Moreover, these $CD8^+$ T-cells produced IFN-γ, an essential cytokine for host protection against viral infection. As both $CD4^+$ and $CD8^+$ T-cell populations seemed to be activated, it is interesting to consider their immunologic functions. The $CD8^+$ T-cell subset activated after immunization may act through cytotoxic activity against infected cells and indirectly through secretion of IFN-γ as described in many viral infections [25]. The effector function of $CD4^+$ cells may also act through cytokine production. The cytokine pattern detected could indicate that $CD4^+$ T-cells from the Th1 subset were stimulated and provide help for $CD8^+$ T-cells. $CD4^+$ T-cells could also participate in the immunological process by producing IL-2, a cytokine which also interferes with viral replication [26].

Experimental evidence suggests that circulating antibodies are the major effector mechanisms for protection against papillomavirus infection. In animal models, antibodies raised

against recombinant VLPs protect against recurrent papillomavirus infection and the spread of infection [18–21]. As a high level of anti-HPV antibodies has been induced in this study by HPV 16 VLPs (data not shown) it could be suspected that $CD4^+$ T-cells provide help for the production of antibodies to several B-cell epitopes present on the L1 protein. Moreover, T lymphocytes primed with L1 capsid proteins can play a role *in vivo* in the control of HPV infection in the host. To demonstrate the *in vivo* role of both $CD4^+$ and $CD8^+$ T-cell subsets in the outcome of virus infected cells, it is necessary to devise an HPV pathogenic model in immunocompromised mice grafted with infected tissue, as already described by others [15, 16] and to perform adoptive transfer experiments as for other viral infections [27, 28].

E6 and E7 proteins of HPV 16 which are produced by tumour cells infected by HPV's induce humoral and cellular responses in mice and could prevent the development of tumoral lesions [7, 29, 30]. In contrast, it is expected that the cellular immunity induced by L1 HPV virus-like particles will have no or poor effects on tumor development since it is accepted that L1 protein is not expressed or expressed at low level in such lesions. However, L1 protein is produced in low grade lesions [31] and thus a cellular immunity against L1 could play a role in the elimination of cells infected by viruses which could escape antibody neutralization. It could be expected that $CD8^+$ T-cells infiltrating infected tissues express cytolytic activities, produce IFN-γ and thus participate in the clearance of the infected cells.

Taken together, the results obtained with L1 virus-like particles confirmed that they are the immunogen of choice for a prophylactic HPV vaccine.

Materials and methods

Preparation of viral antigen and immunization protocol

Recombinant HPV 16 VLPs were prepared as described previously [32,33]. HPV 16 VLPs were purified from Sf 21 cells infected with Ac/16 L1 recombinant baculovirus. Briefly, HPV DNA was extracted from HPV 16 infected cervical cells obtained by scraping. HPV 16 L1 ORF was amplified by polymerase chain reaction (PCR). The PCR product was then cloned into the pBlue Bac III vector. The expression vector obtained was used to co-transfect Sf-21 cells with *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) genomic DNA. After selection of the Ac/16 L1 recombinant baculoviruses by end point dilution, they were used to infect Sf 21 cells. Nuclei of infected insect cells were sonicated and the lysate was loaded on 40% sucrose.

HPV was concentrated by ultracentrifugation (28 000 rpm, 3h, Beckman, SW 28, U.S.A.) and the resuspended pellet was loaded onto a CsCl gradient. After ultracentrifugation at 26 500 rpm for 20 h, the gradient was collected and the different fractions were tested for density and the presence of VLPs by electron microscopy and ELISA. Viral particles were observed in CsCl gradient fractions with densities ranging from 1.27 to 1.30. The positive fractions were pooled, inactivated with formaldehyde and adsorbed on aluminium hydroxide as adjuvant. A vaccine dose (200 µl) contained 5 µg of protein.

The viral antigen used for lymphoproliferation stimulation was further purified though a second sucrose (40%) and a second isopycnic CsCl-gradient. Two negative control antigens were used in order to demonstrate that cell-mediated immunity did not result from non-specific immune stimulation. Such lymphocyte stimulation could be caused by immunogenic insect cell components (which could contaminate the preparation) and by proteins associated with virus-like particles, such as heat shock proteins which have been shown to associate with polyomavirus capsid proteins [34]. The first (WT), corresponds to a CsCl gradient fraction obtained from Sf21 cells infected with wild type baculovirus. The second negative control antigen comprises human hepatitis B core antigen (HBc) virus-like particles, prepared according to the same purification procedure from Sf21 cells, but infected with a recombinant baculovirus encoding the HBc gene.

Seven to 8 week-old BALB/c female mice (IFFA-credo, St Germain L'Arbresle, France) were subcutaneously immunized weekly for 3 weeks, and were given a booster dose 2 weeks later.

Control mice received aluminium hydroxide alone in the same conditions. Five mice were used in each experimental group and the results were representative of three independent experiments.

Lymphocyte proliferation and generation of lymphokine-containing supernatants

Mice were killed 8 days after the last immunization. Splenocytes were isolated and contaminating red blood cells were lysed by hypotonic shock with a 0.83% ammonium chloride solution.

Cells were resuspended in RPMI-1640 HEPES culture medium (Gibco, Paisley, U.K.) containing 5% fetal calf serum (Eurobio, Casulis, France) L-glutamine (2mM), 100IU/ml penicillin (Sigma, Saint Quentin Fallavier, France) and 100 μg/ml streptomycin (Sigma).

For the lymphoproliferation assay, 2.10^5 cells were seeded in triplicate into a flat-bottomed 96-well culture plate (Falcon, Cockeysville, MD), in the presence of various dilutions of purified HPV 16 L1 VLPs (0.8 to 100 μg/ml), WT antigen (0.8 to 100 μg/ml), HBc VLPs (0.2 to 100 μg/ml), bovine serum albumin (BSA, 0.08 to 100 μg/ml, Sigma, Saint Quentin Fallavier, France) or Concanavalin A (Con A, 0.2 to 10 μg/ml, Sigma, Saint Quentin Fallavier, France) and incubated for 5 days at 37°C in a humidified 5% CO_2 atmosphere. Following incubation 18.5 KBq of ^{3}H-thymidine (Specific activity, 37 GBq/mmol; NEM, Dupont de Nemours, Wilmington, DE) were added for 18h and incorporation was measured by liquid scintillation counting. Results, expressed in cpm, are the mean of three wells.

For generation of lymphokine-containing supernatants 2.10^6 cells were seeded in duplicate in flat-bottomed 24-well culture plates (Falcon, Cockeysville, MD) in the presence or absence of 15 μg/ml purified HPV 16 L1 VLPs or HBc VLPs. For lymphokines generated by CD8 T-cells, thioglycolate-elicited macrophage cells were used as target cells. Culture supernatants were collected at 24h, 48h and 72h intervals.

Purification of CD8 T-cells by magnetically activated cell sorting

Thirty million purified lymphocytes were incubated with rat antimouse CD8 monoclonal antibody (Ab) (Pharmingen, San Diego, U.S.A.) for 30 mn at 4°C followed by 15 mn incubation at 4°C with goat anti-rat lgG conjugated with magnetically activated cell sorting (MACS) supermagnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). These complexes were then applied to a prewash column with PBS containing 10% fetal calf serum (PBS–FCS) on the mini-MACS system (Miltenyi). Non-adherent CD8-cells were collected by passing them through 10% PBS–FCS and then reapplied three times onto the column. The separation was then removed from the mini-MACS and $CD8^+$ cells were eluted by washing with PBS-FCS 10% and resuspended in RPMI 1640-HEPES culture medium. The mini-MACS separation fractions were analysed by flow cytometry. These mini-MACS separations allowed a purity of >90% for each lymphocyte cell subset examined in this study as ensured by flow cytometry.

Immunofluorescence labelling and flow cytometry

Helper/inducer and suppressor/cytotoxic T cells were analysed using rat monoclonal antibodies (anti L3T4 ($CD4^+$) lymphocytes) GK 1.5 [35], anti-Lyt-2 ($CD8^+$ lymphocytes) H35.17.2 [36] and anti-Thy1-2 (predominantly T lymphocytes) (Pharmingen, San Diego, U.S.A).

A goat polyclonal serum raised to mouse lgG was used to label surface immunoglobulin positive cells (lgS^+), mainly B cells.

Immunolabelling and flow cytometry were performed as previously described [37]. Briefly, splenic cells were incubated (1h, 4°C) with the appropriate antibodies. After washing, cells were stained (45 mn, 4°C), with a fluorescein-conjugated rabbit anti-rat lg (diluted 1/100) or rabbit anti-goat antibody (diluted 1/80) (Nordic Immunology, Tilburg, The Netherlands). Cells were then washed again and phenotype analysis was performed by cytometry with a FACSsort (Becton Dickinson, Le pont de Claix, France).

Results from five mice treated independently from at least two separate experiments, were analysed by using the Mann–Whitney U-test. $P \leq 0.05$ was considered as significant.

Cytokine analyses in culture supernatants

Interferon γ (IFN-γ), interleukin 2, 4 and 5 (IL-2, IL-4, IL-5) were assessed by a sandwich ELISA according to the manufacturer's recommendations using an anti-mouse IFN-γ, anti-IL-2, anti-IL-4, and anti-IL-5 antibody (Genzyme, Boston, MA). Cytokine concentrations were determined by reference to standard curves constructed with fixed amounts of mouse recombinant IL-2 (Genzyme), IL-4 (Pharmingen, San Diego, U.S.A.), IFN-γ (Genzyme), or IL-5

(standard lyophilizated Th2 supernatant from DNAX Research, Palo Alto, CA). Each cytokine secretion was tested in culture supernatants at 24h, 48h, and 72h. For each cytokine, the results shown are expressed as the maximum of secretion.

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